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Association between DNA Methylation and Oxidative Stress Index in Type 2 Diabetes Patients with Retinopathy

A Thesis

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BY

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بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

﴿قَالُواْ سُبۡحَٰنَكَ لَا عِلۡمَ لَنَآ إِلَّا مَا عَلَّمۡتَنَآۖ إِنَّكَ أَنتَ ٱلۡعَلِيمُ ٱلۡحَكِيمُ﴾

صدق الله العلي العظيم

سورة البقرة: الاية ﴿٣٢﴾

Dedication

To the one who sacrificed precious things for the sake of my attaining a high degree of knowledge and left before seeing the fruit of my father's planting may God have mercy on him

To the source of love, altruism, and generosity, my mother, may God prolong her life

To the one who left a longing that the years do not hide, the most precious one I lost, my brother, may God have mercy on him

To my support, my strength, and my sanctuary My brothers and sisters

To everyone who helped me in my scientific career

Farah

2023

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I also extend my sincere thanks to my professors, the esteemed members of the Discussion Committee, for the effort they took in reading my humble letter and enriching it with their suggestions. the value. I also extend my sincere thanks to the consultant at Mahaweel General Hospital and diabetic patients for their cooperation with me. I have not forgotten and will not forget to extend my deepest thanks and gratitude to everyone who lit a candle in the paths of our knowledge and to those who stood on the platforms and gave from the outcome of their thought to illuminate our path to the honorable professors.

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Summary

In the study, we aimed to find out whether overall DNA methylation is a factor in diabetic retinopathy. The study was designed to evaluate total 5- methyl cytosine (5mC%) levels and study the correlation between these levels and some physiological parameters in patients with type 2 diabetes (T2D) and diabetic retinopathy compared to the control group.

The study included collecting 120 donors, including 40 type 2 diabetes patients 40, diabetic retinopathies and 40 control. The present study involved the physiological study and epigenetic study; the physiological study included some important characteristics of both diabetic and healthy control such as age, body mass index (BMI), sex and smoking status. Also, it is involved a physiological evaluation of some diabetes-related parameters: fasting blood glucose (FBG), glycated hemoglobin (HbA1C), insulin, insulin resistance (IR) and insulin sensitivity (IS). As well as the evaluation of some oxidative stress parameters, which included: total antioxidant capacity (TAC), reactive oxygen species (ROS),and oxidative stress index (OSI); and the assessment of the inflammatory factor: human vascular endothelial growth factor A (VEGF-A). The epigenetic study included the assessment of global DNA methylation in study.

The results showed a significant ($p \leq 0.05$)increase in the mean of age and BMI for T2D patients compared with healthy subjects. For sex, identical numbers of males and females were taken.

Statistical analysis showed that FBG, HbA1C, insulin, IR, TAC, ROS, OSI, VEGF-A significantly are ($p \leq 0.05$) increased in T2D patients, while IS decreases significantly($p \leq 0.05$) as compared with control subjects. Comparison the physiological parameters showed that significant increase($p \leq 0.05$) in FBG, insulin, ROS, OSI, VEGF-A in retinopathy patients compared to T2D patients.

The distribution of the retinopathy and T2D patients according to their age showed the highest percentages in the (35-44, 45-54, 55-64 and≥60 years).The statistical analysis showed that in both groups (diabetic retinopathy and T2D) FBG have a significant ($p \leq 0.05$) increase in55-64 and≥65age groups compared with others, while HbA1C, insulin and IR showed a significant increase in≥60age group compared with others. ROS and OSI hade asignificant increase within age categories, the highest levels were in ≥60 for retinopathy and in 55-64 and≥65 for T2D.

According to BMI, the highest levels of FBG, Insulin and IR were in obesity and morbid obesity in both retinopathy and T2D. Highest levels of HbA1C in morbid obesity were compared with other BMI groups in T2D only. TAC recorded a significant increase in normal and overweight groups in retinopathy; only in normal weight group in T2D. The highest levels of ROS and OSI were in obesity and morbid obesity in both groups. VEGF-A significantly increased in($p \leq 0.05$) obesity and morbid obesity groups compared with other groups in both patient's groups.

The effect of gender on studied physiological markers showed a significantly increased($P \leq 0.05$)in levels of HbA1C in females under go from retinopathy only. TAC significantly increased in males, while ROS and OSI showed a significant increase($p \leq 0.05$) in females in T2D only. VEGF showed a significant increase in females compared with males only in retinopathy.

According to smoking habit, HbA1C, IR, ROS, and VEGF showed a significant($P<0.05$) increase in smoker patients compared to non-smoker patients, while IS and TAC significantly increase in non-smoker for retinopathy. For T2D, a significant increase($p \leq 0.05$) in FBG, IR, ROS, and OSI in smoker patients compared with non-smoker was recorded, while TAC was significantly increase($p \leq 0.05$) in non-smoker.

As for the epigenetic study, global DNA methylation analysis revealed that patients with diabetes (both T2D and those with retinopathy) had a significantly increase($p \leq 0.05$) in mean levels of 5methyl cytosine % compared to healthy subjects. Also, a significant increase($p \leq 0.05$)was recorded in diabetic retinopathy compared with T2D patients.

According to gender, study showed a significant increase($p \leq 0.05$) in 5mC% DNA methylation levels in females control. in females patients compared to males, also found within the retinopathy group: methylation levels increased($p \leq 0.05$) in females compared to males.

According to smoking, all groups showed a significant increase($p \leq 0.05$) in average of 5mC% levels in smoker patients compared to non-smoker patients.

Correlation analysis showed asignificant($p \leq 0.05$) positive correlation in the levels of 5methyle cytosine % in all studied groups

With regard to the association between physiological markers and DNA methylation, study found significant positive correlation between DNA methylation and HbA1C in control subjects. In patients, DNA methylation had a significant positive($p \leq 0.05$) association with FBG, HbA1C and IR as well as a significant negative association with IS.ROS and OSI showed significant positive correlation with DNA methylation in patients, VEGF positively correlates with 5mC% in both control and patients. DNA methylation significantly associated with FBG, HbA1C, IR, as well as a significant negative association with IS in both retinopathy and T2D. ROS, OSI and VEGF showed a significant positive correlation with5mC% in both retinopathy and T2D.

We conclude that increased levels of abnormal DNA methylation in the blood can be used as a biomarker to diagnose diabetic retinopathy earlier than current clinical methods.

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List of Abbreviations

Abbreviation	Full term
ADA	American Diabetes Association
AGEs	Accumulation of Glycation End Products
BMI	Body Mass Index
DM	Diabetes Mellitus
DNMTs	DNA Methyltransferases
DPPH	Diphenylpicrylhydrazyl
ELISA	Enzyme-Linked Immunosorbent Assay
FBG	Fasting Blood Glucose
FRAP	Ferric Reducing Ability of Plasma

HbA1C	Glycated Hemoglobin
IDF	The International Diabetes Federation
IR	Insuline Resistance
IS	Insuline Sensitivity
Lnc-RNA	Long non Coding-RNA
NADPH	The Nicotinamide Adenine Dinucleotide Phosphate
NIDDM	Non Insulin-Dependent Diabetes Mellitus
OSI	Oxidative Stress Index
PKc	Protein kinase C
RNAi	RNA Interference
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SAM	S-Adenosylmethionine
SiRNA	Small Interfering RNA
T1D	Type1 Diabetes
T2D	Type 2 Diabetes
TAC	Total Antioxidant Capacity
TETs	Ten-Eleven Translation
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

Chapter One
Introduction

1.1 Introduction

Diabetes mellitus (DM), which is expected to affect about 750 million people by 2030, is one of the chronic diseases with the highest social and economic consequences and continues to pose a challenge to public health policy (Forouzanfar *et al*., 2016). Diabetes is prevalent worldwide and one of the most important causes of increasing mortality and morbidity. For that reason, DM is a very common disorder that is seen so frequently during every day clinical practice all over the world (Xu *et al*., 2018). The prevalence of diabetes considerably rises with time, primarily due to an increase in type 2 diabetes (T2D) incidence (Lovic *et al*., 2020).

Insulin resistance is belief to precede the emergence of T2D by 10 to 15 years (Henstridge *et al*., 2019). The development of IR typically results in a compensatory boost in endogenous insulin production, Increased levels of insulin, an anabolic hormone, is closely related with IR and result in weight gain which, in turn, augment the IR (Laursen *et al*., 2019). As this vicious cycle continues, beta cell activity loses the ability to meet the insulin demand created by IR, resulting in hyperglycemia (Freeman and Pennings, 2022). With ongoing mismatch between insulin production and insulin demand, glycemic levels rise become as a chronic hyperglycemia that predisposing individuals to long term microvascular (nephropathy, neuropathy, and retinopathy), and macro vascular (cerebrovascular, cardiovascular and hypertension) complications and non-traumatic lower extremity amputations worldwide (Lowe *et al*., 2014).

Retinopathy is an important complication of T2D that continues to be the cause of preventable and treatable vision loss all over the world, it has been suggested that the correlation between hyperglycemia, changes in the redox homeostasis, and oxidative stress are the key events in the pathogenesis of retinopathy (Kowluru,2003; Ayan *et al*., 2023). In comparison to other tissues, the retina has the highest oxygen absorption and glucose oxidation due to its high

amount of polyunsaturated fatty acids, this phenomenon increases the retina's sensitivity to oxidative stress (Anderson *et al*.,1984).

In retinal tissues, hypoxia triggered by microvascular occlusion leads to release of vasogenic mediators like vascular endothelial growth factor (VEGF), and thus to abnormal vascular pathologies (Fu *et al*., 2016).

Epigenetic research is currently one of the most pertinent hot issues due to the reversible nature of its mechanisms, flexibility, and phenotypic effect, which offers new therapeutic options for retinal disorders (Wu *et al*., 2021).

Methylation of DNA involves the covalent addition of a methyl group to carbon C5 of cytosine nucleotides to create 5-methylcytosine (Wang *et al*., 2020). More recently, it was proved that methylation of DNA is involved in T2D early stages showing a mechanism of metabolic memory (Kumari *et al*., 2020). Also, it is found that 5mCmechanism closely related to normal and pathological development of the human retina (Law and Holland,2019). Suggesting that a high DNA methylation status may be a potential risk factor for diabetic retinopathy (Zhang *et al*., 2017). It is involved in important pathophysiological processes, including embryonic development, stem cell differentiation, tumorigenesis and aging (Zhu *et al*., 2021).

Excessive ROS can damage cell structures, including lipids, membranes, proteins, and nucleotides, so it can affect and triggered methylation of DNA (Augustine *et al*., 2021). On the other hand, DNA methylation promotes oxidative stress and ultimately contributes to the development of retinopathy (Kowluru and Shan, 2017).

1.2 The Aim of the study

Describe the association between global DNA methylation and T2D with and without retinopathy, additionally, investigate the relationship between

global DNA methylation with some liability traits, oxidative stress index and vascular endothelial growth factor.

Chapter Two

Review of literatures

2. Review of literatures

2.1 Diabetes Mellitus

Diabetes mellitus (DM) is a significant public health problem that affects more than 400 million people globally (Khursheed *et al*., 2019). Due to the significant human and financial burden it places on society, diabetes is one of the biggest universal health emergencies of the twenty-first century (Atlas, 2015). Over the past four decades, Iraq has experienced a sharp rise in the prevalence of diabetes, which now stands at 20% (Mansour and Al Douri, 2015). Due to changes in food and lifestyle patterns, population aging, urbanization, and a genetically predisposed environment, DM is a huge burden on society (Nolan *et al*., 2011).

Diabetes mellitus is a systemic disease in which blood glucose levels become chronically, and often severely, elevated either because insulin is not secreted from the pancreatic islet cells (type 1 diabetes), or because the insulin that is secreted is, for a variety of reasons, less than normally efficacious (type 2 diabetes) (Frank¸ 2015). The majority of Non-communicable diseases are metabolic diseases, and by 2045, the International Diabetes Federation (IDF) projects that there will be more than 693 million individuals with diabetes worldwide (IDF, 2019). A persistent rise in blood sugar levels or diabetes is progressively brought on by either IR or insulin insufficiency brought on by the cumulative decline in ß-cell function (or both) (Chen *et al*., 2017). Diabetes was described by the American Diabetes Association (ADA) as a complicated chronic illness requiring ongoing medical attention as well as multifactorial risk-reduction techniques beyond glyceamic management, the risk of long-term problems must be reduced through ongoing patient self-management education and assistance (ADA, 2016).

2.1.1 Classification of Diabetes

Diabetes has been traditionally classified into T1D and T2D, over the past decades, the diabetes concept has grown to the realization that there are different

overlapping contributions from genetics and the environment that can lead to manifestations of various forms of DM (IDF, 2019).

The ADA classified DM into several types according to the disease etiology and not according to the treatment form, which includes the following types (ADA, 2014):

1. **Type 1**Diabetes: β-cell destruction, usually leading to absolute insulin deficiency.
2. **Type2** Diabetes (which may range from predominantly IR with relative insulin deficiency to a usually secretory defect with IR).
3. **Gestational diabetes mellitus**
4. **Other specific types**

A. The genetic defects of β-cells function

B. The genetic defects in the action of insulin

C. Diseases of the exocrine pancreas

D. Endocrinopathies

E. The medication or chemical induced

F. The infections

2.2 Type 2 Diabetes

Type 2 diabetes is a widespread condition that posses a severe threat to world health, world Federation for Diabetes (IDF, 2014). Type 2 diabetes formally known as the non-insulin-dependent diabetes mellitus (NIDDM), is a metabolic disorder characterized by chronically hyperglycemia, and it develops due to IR as well as reduced insulin secretion (Kahn *et al.*, 2014).

Type 2 diabetes accounts for around 90% of all cases of diabetes, in T2D, IR is characterized by a decreased responsiveness to insulin, in order to maintain glucose homeostasis at this stage, insulin is inefficient, which is first countered by an increase in insulin secretion, however, with time, this increase in insulin production declines, leading to T2D, people over 30 years old are most likely to

develop T2D (Goyal and Jialal, 2022). A multifactorial disease resulting from the interaction between genetic predispositions and environmental risk factors such as a sedentary lifestyle, nutritional imbalance, stress, and environmental pollutants such as bisphenols, dioxin and pesticides (Lin and Yin, 2022). Prolonged over nutrition(particularly the excess of fatty acide) leads to chronic reactive oxygen species and reactive(ROS) nitrogen species production(RNS), which promotes oxidative stress in cells, tissues, and organs, lipotoxicity-induced oxidative stress results in damage to cell membranes, DNA, and proteins, as well as modulation of the activity of transcriptional factors through redox chemistry, including NF-κB, leading to chronic inflammation, IR, and cell apoptosis (Newsholme *et al*., 2009).

It has been shown that a significant fraction of T2D patients eventually need insulin therapy as a result of oral hypoglycemic drugs' failure to control blood glucose levels in these patients (Solis-Herrera *et al*., 2018). Long-term harm, dysfunction, and failure of certain organs, particularly the heart, blood vessels, kidneys, nerves, eyes, and kidneys, are associated with the chronic hyperglycemia of diabetes (ADA, 2010).

According to (Huether *et al*., 2014) complications of diabetes include the following:

- Eyes: Retinopathy and Cataracts.
- Central and Peripheral Nervous System: Neuropathy, and decreased cognition.
- Circulatory: Heart disease, cerebrovascular accident, peripheral vascular disease, and hypertension.
- Liver: Steatohepatitis and biliary disease.
- Gastrointestinal Tract: Gastroparesis.
- Kidneys: Nephropathy and chronic kidney disease.
- Hematologic System: Oxidative stress, immunosuppression, infection, and cancer.

Type 2 diabetes management differs dramatically between Iraqi public and private sectors; this variability is due to treatment access discrepancy (Abusaib *et al*., 2020).

2.2.1 Hyperglycemia

Hyperglycemia is a clinical manifestation in diabetes (Ola *et al*., 2012). It plays an important role in the pathogenesis of microvascular damage in the retina, multiple metabolic pathways have been linked to vascular damage caused by hyperglycemia, including the polyol pathway, the accumulation of glycation end products, the protein kinase C pathway, and the hexosamine (Brownlee, 2005). Retinal ischemia/hypoxia leads to upregulation of VEGF through activation of hypoxia-inducible factor 1(Huang *et al*., 2015). Other evidence suggested that phospholipase A2's elevation under the diabetic condition also triggers upregulation of VEGF (Lupo *et al*., 2013).

Hyperglycemia serves as the starting point of aberrant DNA methylation in diabetes (Cai *et al*., 2020). First, in the initial stages of DM, reestablishment of good glycemic control prevents retinal mitochondria from being compromised when the activation of DNA methyltransferases and Ten-eleven translocation dioxygenases and the methylation of mtDNA and nDNA remain unchanged, this shows that by controlling blood glucose early and maintaining it at good levels, the DNA methylation mechanism does not damage the retina of diabetic patients, additionally, even if patients cannot or do not have strict glyceamic control in the initial phase of DM, long-term strict glyceamic control can still improve abnormal methylation status and ultimately delay or stop the development of retinopathy (Mishra and Kowluru, 2016).

2.2.2 Pathophysiology

Type 2 diabetes is a heterogeneous and multifactorial disease, which affects whole body physiology(Chatterjee *et al*., 2017). The pathophysiology of diabetes is related to the levels of insulin within the body, and the body's ability to utilize

insulin, there is a total lack of insulin in T1D, while in type 2 diabetes, the peripheral tissues resist the effects of insulin, normally, the pancreatic beta cells release insulin due to increased blood glucose concentrations, the brain in order for normal functions to occur continually requires glucose, hypoglycemia, or low plasma glucose levels, is usually caused by drugs used in the treatment of diabetes, including insulin and oral anti-hyperglycemics (Moini, 2019). IR is a condition in which insulin in the body does not exert sufficient action proportional to its blood concentration, the impairment of insulin action in major target organs such as liver and muscles is a common pathophysiological feature of T2D, IR develops and expands prior to disease onset (Kohei, 2010). T2D pathophysiological process is complex where disorganization of gene expression is predicted and this will lead to discompose of variable physiological processes in tissues which participate in glucose homeostasis (Das and Sharma, 2014).

The individuals with impaired glucose tolerance have hyperglycemia in spite of having highest levels of plasma insulin, indicating that they are resistant to the action of insulin, in the progression from impaired glucose tolerance to DM, the level of insulin declines indicating that patients with NIDDM have decreased insulin secretion, IR and insulin deficiency are common in the average NIDDM patients (Holt, 2004). IR is the primary cause of NIDDM, however some researcher contend that insulin deficiency is the primary cause because a moderate degree of IR is not sufficient to cause NIDDM (Raju and Raju, 2010).

Insulin is a hormone produced by the pancreatic β-cells and is the key hormone for the regulation of blood glucose, it stimulates uptake of glucose from the blood in the muscle and fat tissue and store the glucose as glycogen in the liver and muscle cells, and esterification of fatty acids occurs in adipocytes, in addition, insulin inhibits the breakdown of proteins, the hydrolysis of triglycerides and the production of glucose from amino acids, lactate and glycerol, glucagon, which is also secreted by the endocrine pancreas, has the opposite effects to that of insulin, the hormone causes the liver to convert stored glycogen into glucose, thereby

increasing the level of blood glucose, besides, glucagon stimulates insulin secretion, so that glucose can be used by insulin-dependent tissues, hence, glucagon and insulin are part of a feedback system that keeps blood glucose at the normal level (Jones *et al*., 2012) .

2.3 Retinopathy

Diabetic retinopathy is defined as damage to the retina's micro vascular system caused by persistent hyperglycemia, which can result in blindness (Mclnnes and Schett, 2011).

Diabetic Retinopathy is a common complication of DM, which causes lesions on the retina that effect vision (Alyoubi *et al*., 2020).

The possibility of retinopathy presence increases for diabetes patients who suffer from the disease for a long period, retina regular screening is essential for diabetes patients to diagnose and to treat retinopathy at an early stage to avoid the risk of blindness (Chakrabarti *et al*., 2012). Retinopathy is detected by the appearance of different types of lesions on a retina image, these lesions are microaneurysms, haemorrhages, soft and hard exudates (Taylor and Batey, 2012).

Retinopathy is characterized by progressive changes in the retinal microvasculature, leading to areas of retinal ischemia, increased vascular permeability, and pathological intraocular proliferation of retinal vessels. Complications are associated with macular edema and uncontrolled neovascularization (Duh *et al*., 2017). Studies, however, have demonstrated that retinal neurodegeneration is a critical feature associated with the progression of the disease and that early retinal neuronal injury actually precedes microangiopathy (Villarroel *et al*., 2010; Simó *et al*., 2018).

The primary underlying mediator of diabetes complications is the damage due to hyperglycemia and other excess fuels caused by reduced insulin or reduced insulin effect, the development and progression of complications depends on the

interplay between genes, epigenetic changes due to the environment, IR, immune dysregulation and inflammation, fuel excess (Schwartz *et al*., 2017).

This damage is accomplished by modulation of redox regulators and epigenetic changes in these susceptible cells and tissues that is encompassed (in part) by Brownlee's Unified Theory of "Diabetic" Complications (Shah and Brownlee, 2016) .

This dysfunction leads to increased inflammatory cytokines and chemokine production, aberrant growth factor signaling and ROS resulting in neuro-glial degeneration and vascular dysfunction and its associated alteration of the blood-retinal barrier, hypoxia, vascular permeability resulting in edema and angiogenesis (Rübsam *et al*., 2018).

2.3.1 Mechanism of Diabetic Retinopathy

A range of studies have described the biochemical mechanisms in the development of retinopathy, however, there is no mechanism that can be considered to bein place, all forms of DM are characterized by hyperglycemia, IR, relative or absolute deficiency in insulin action, and the appearance of diabetic-specific pathology in the retina (Safi *et al*., 2014).

Insulin resistance, defined as the condition where insulin responsive tissues fail to increase glucose uptake in response to physiological concentrations of insulin (Reaven, 2005). IR is characterized by high levels of circulating insulin, due to increased insulin secretion by β cells as a compensatory mechanism to counteract IR. A number of factors including IR, as described above, genetic predisposition, glucotoxicity, lipotoxicity, increased ROS, endoplasmic reticulum , stress and elevated intracellular calcium (Poitout and Robertson, 2008) , contribute to β-cell dysfunction, which is a prerequisite for the development of T2D (Cerf, 2013). IR in peripheral tissue, and decreased insulin secretion due to pancreatic β-cell dysfunction is accepted to play a major role (Tripathy and Chavez, 2010).

Molecular and biochemical mechanisms that have been implicated in diabetic retinopathy are increased flux of glucose through the polyol and hexosamine

pathways, activation of protein kinase C (PKC), and increased advanced glycation end product formation (Brownlee, 2001).

The polyol pathway is a two-step metabolic pathway in which glucose is reduced to sorbitol, which is then converted to fructose, several biochemical and molecular studies implicate the polyol pathway as a reasonable and significant contributor to diabetic retinopathy and other complications of diabetes, retinal endothelial cells of both rat and human showed aldose reductase immunoreactivity and human retinas exposed to high glucose in organ culture increased the production of sorbitol by a degree comparable to that observed in the rat, such excess aldose reductase activity can be a mechanism for human diabetic retinopathy (Dagher *et al*., 2004).

Diabetes is accompanied by an increased diabetic retinopathy (Ezquer *et al*., 2014).

Epigenetic mechanisms including DNA methylation, histone modifications, and miRNAs and long non-coding RNA (lnc-RNA) regulation contribute to the dysregulation of signaling pathways involved in oxidative stress, inflammation, apoptosis, and aging, and modulate the expression of several key genes in DM (Gilbert and Liu, 2012).

Epigenetic mechanisms include DNA methylation, lysine methylation, histone methylation, histone phosphorylation, RNA interference (RNAi) and genomic imprinting (Sharma *et al*., 2009). Lysine methylation refers to a process whereby enzymes called lysine methyltransferases catalyse the addition of one or more methyl groups from S- adenosyl-L-methionine to the $\varepsilon_{\text{ٍ}}$-amino group of a lysine residue (Qian and Zhou, 2006). Histone methylation occurs when a methyl group is attached to the amino acids of histone proteins on nucleosomes (Szyf, 2009).

Histone phosphorylation refers to the addition of a phosphate group to histone proteins and it is a key process which regulates chromatin structure (Rossetto *et al*., 2012). RNAi is an epigenetic mechanism involved in gene expression control

and its mechanisms include RNA induced silencing complex, small interfering RNA (siRNA), lnc-RNAs and microRNAs (Fatica and Bozzoni, 2014). Genomic imprinting is an epigenetic mechanism in which the expression of a gene is restricted to one of the parental alleles (Sharma *et al*., 2009).

DNA methylation has been observed to be a highly effective mechanism (Szyf, 2009). DNA methylation alters protein binding to target sites on DNA, leading to transcriptional silencing on genes and interference with heterochromatin formation, the silenced states of the genes can be inherited throughout cellular divisions and eventually affect the phenotype, hence the development of disease (Heyn and Esteller, 2012).

One suggested mechanism by which single nucleotide polymorphisms (SNPs) change gene expression is through epigenetics via the introduction or removal of CpG sites, which are potential DNA methylation sites. In addition, these CpG-SNPs may affect the expression of their target gene(s) through other mechanisms, such as interfering with the binding of certain proteins (Dayeh *et al*., 2013). Also affecting intragenic DNA methylation through exonic splicing enhancers. Previous studies have shown that intragenic DNA methylation plays a key role in the regulation of alternative splicing (Karambataki *et al*., 2017).

2.3.2 Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor-A (VEGF-A) is key in the development and progression of retinopathy, granting a conformational alteration to the tight junctions of retinal vascular endothelial cells and working in large part to boost the vascular permeability and hike vessels proliferation (Witmer *et al*., 2003). VEGF is a multifunctional growth factor implicated in embryonic development, and it is a powerful angiogenic agent in physiological and pathological neovascularization (Grassi *et al*., 2019). The VEGF belongs to the endothelial growth factor family (VEGFs) (Di Venere *et al*., 2017). The VEGF family is composed of five

structurally related ligands: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor, these ligands bind in an overlapping pattern to three tyrosine kinase receptors (VEGFR-1, VEGFR-2, and VEGFR-3) (Ferrara, 2004).

Population-based studies suggest that one-third of the diabetic patients have signs of retinopathy and one-tenth have vision-threatening states of retinopathy, such diabetic macular edema and proliferative diabetic retinopathy (Lamoureux and Wong, 2011). VEGF-A plays a central role in both diabetic macular edema and Proliferative diabetic retinopathy development (Antonetti *et al*., 2012).

Vascular endothelial growth factors are a subfamily of growth factors that function as signaling proteins for both vasculogenesis (the de novo formation of the embryonic circulatory system) and angiogenesis (the growth of blood vessels from pre-existing vasculature). VEGF is secreted primarily from retinal pigmented epithelial cells, pericytes, astrocytes, müller cells, glial cells, and endothelial cells (Tischer *et al.,* 1991).

2.4 Risk Factor for Type2 Diabetes and Diabetic Retinopathy

Type 2 diabetes risk factors include a complex combination of genetic, metabolic and environmental factors that interact with one another contributing to its prevalence (Schellenberg *et al*., 2013). The risk factors of retinopathy can be broadly classified into modifiable (i.e., hyperglycaemia, hypertension, hyperlipidaemia, obesity, and cigarette smoke) and non-modifiable factors (i.e., duration of diabetes, puberty, pregnancy and genetic susceptibility), these risk factors are also involved in the development of both diabetic nephropathy, neuropathy and macrovascular complications (Sasso *et al*., 2021). Globally, the incidence and prevalence of T2D are found to vary widely depending on ethnicity and geographical region with Japanese, Hispanics and Native Americans having the highest risks (Liu *et al*., 2009). Obesity (body-mass index [BMI]≥30 kg/m$_2$) is the strongest risk factor for T2D (Bellou *et al*., 2018), also it is associated with

metabolic abnormalities resulting in IR (Sinha *et al*., 2002). There is an inverse linear relationship between BMI and the age at diagnosis of T2D (Hillier and Pedula, 2003). There are three primary benefits of physical activity on the delay of T2D onset, first, the contraction of skeletal muscle cells induces an increase in blood flow into the muscle, enhancing glucose uptake from plasma (Venkatasamy *et al*., 2013). Second, physical activity reduces the notorious intra-abdominal fat, which is a known risk factor that promotes IR (Strasser, 2013). Finally, moderate-intensity exercise has been shown to improve glucose uptake by 40% (Ross, 2003). Physical activity improves glucose uptake and insulin sensitivity but it can also improve or even reverse inflammation and oxidative stress, which are T2D predisposing factors (Venkatasamy *et al*., 2013). An increase in body fat is generally associated with an increase in risk of metabolic diseases such as T2D, hypertension and dyslipidaemia(WHO, 2016). Abdominal obesity may cause proinflammatory chemicals to be released by fat cells, these chemicals could decrease the insulin sensitivity by disrupting the function of insulin-responsive cells and their ability to respond to the insulin (Freemantle *et al*., 2008).

Smoking is another risk factor for T2D and diabetic retinopathy, according to the 2014 Surgeon General's Report, smoking increases the risk of T2D by 30–40% for active smokers compared to non-smokers, suggesting that smoking cessation should be emphasized as an essential public health strategy to combat the global epidemic of diabetes (US Department of Health and Human Services, 2014). Cigarettes and other smoking products contain a mixture of chemical additives that can affect metabolic health, nicotine is one of the most widely consumed biologically active substances (Kimura *et al*., 2016).

Nicotine has been shown to directly alter glucose homeostasis (Epifano *et al*., 1992), suggesting an important role for this additive in the development of T2D (Chiba and Masironi,1992). Several biological mechanisms have been

proposed through which smoking may have an effect on the development of diabetes, including inflammation and the effect of nicotine on IR (Xie *et al*., 2009).

However, the exact molecular mechanisms connecting smoking to an increased risk of diabetes remain largely unknown, previous research has established that tobacco smoking has an important role in DNA methylation, the epigenetic mechanism of attachment of a methyl group to a nucleotide (Steenaard *et al*., 2015).

2.5 Oxidative stress in Diabetic Retinopathy

Oxidative stress is defined as an imbalance between the generation and removal of ROS in favor of the oxidants formation, and it is appearing to be raised in a system where the production rate of free radicals is increased and/or the antioxidant mechanisms are reduced (Pieme *et al*., 2017).

Oxidative stress is involved in the pathogenesis of multiple diseases, including diabetes and its complications. Retinopathy, amicrovascular complication of diabetes, is the primary cause of acquired blindness in diabetic patients, increasing data indicate that oxidative stress is involved in the development of retinopathy (Kowluru, 2006; Kang and Yang, 2020). Oxidative stress can both contribute to and result from the metabolic abnormalities induced by hyperglycemia, mainly including the increased flux of the polyol pathway and hexosamine pathway, the hyper-activation of PKC isoforms, and the accumulation of advanced glycation end products (AGEs), moreover, the repression of the antioxidant defense system by hyperglycemia-mediated epigenetic modification also leads to the imbalance between the scavenging and production of ROS (Kang and Yang, 2020).

Excessive accumulation of ROS induces mitochondrial damage, cellular apoptosis, inflammation, lipid peroxidation, and structural and functional alterations in retina; Therefore, it is important to understand and elucidate the oxidative stress-related mechanisms underlying the progress of diabetic

retinopathy (Kang and Yang, 2020). ROS are the active inter-mediates of DNA methylation and can participate in epigenetic processes by nucleophilic substitution reactions (Afanas'ev, 2014). Dnmts, the enzymes that modulate methylation status, are sensitive to redox reactions (Ziech *et al*., 2011). ROS production is able to activate these enzymes, promoting DNA methylation by deprotonating cytosine molecules (Afanas' ev, 2014). Numerous environmental variables like air pollution and tobacco smoking contributes to ROS generation which is supposed to change the pattern of DNA methylation through different mechanisms of oxidative stress (Angelini *et al*., 2017).

Structural changes may both contribute to and result from functional changes, such as altered blood flow, loss of intercellular junctions, and increased vessel permeability, thus, oxidative stress-induced structural and functional changes appear to be highly interrelated in the pathogenesis of retinopathy (Madsen-Bouterse and Kowluru, 2008).

2.5.1 Reactive oxygen species

The term “ROS” includes all unstable metabolites of molecular oxygen (O2) that have a higher reactivity than O2, such as the superoxide radical (O2′•) and the hydroxyl radical (HO•), and non-radical molecules, such as hydrogen peroxide (H_2O_2) (Rahal, 2014). The ROS are free radicals, oxidant molecules that contain one extra electron conferring them great instability and reactivity, by trying to regain stability, they obtain electrons from other molecules in the vicinity, therefore creating an oxidative chain (Packer and Cadenas, 2007).

The free radicals are molecules contain oxygen with an unpaired electron in outer membrane which allow to react with other molecules, on the other hand; free radicals can be causes large chain chemical reactions in the body, which sometimes have harmful effects (Kurutas and Ozturk, 2016).

It is widely accepted that hyperglycemia-induced ROS, that contribute to cell and tissue dysfunction in diabetes (Fatehi-Hassanabad *et al*, 2010). The ROS are generated mainly from two systems: (1) mitochondrial oxidative phosphorylation

and (2) the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system (Dunn *et al*., 2015). One of the ROS-induced dysfunctions in mitochondria is the repression of antioxidant defense capabilities that could lead to enhanced sensitivity of retinal cells to oxidative stress because they cannot scavenge ROS effectively (Kanwar *et al*., 2007).

There is strong evidence that suggests that chronically elevated levels of ROS lead to increased oxidative stress in β-cells, given the ability of ROS to directly damage and oxidize DNA, proteins, and lipids, β-cell functioning is worsened in terms of insulin secretion and action (Cerf, 2020). It has been demonstrated that methylglyoxal, a reactive dicarbonyl metabolite of glucose, together with ROS, have induced apoptosis, the cellular death has also been reported in diabetic retinopathy, in age-related macular degeneration, and in programmed necrosis of the inflammatory cells, with all of them resulting from the action of AGEs, ROS, and methylglyoxal (Jang *et al*., 2017). NADPH oxidase proteins are membrane-associated multiunit enzymes that play a physiological role in response to various factors, as well as pathophysiological roles in diabetic pancreatic β-cells (Elumalai *et al*., 2021).

2.5.2 Total Antioxidant Capacity

Total antioxidant capacity is the primary measurement to evaluate the state and potential of oxidative stress in aging and other age related diseases. estimation of the reducing power/antioxidant capacity the first step in the prediction of oxidative stress in the aging process (Verma and Singh, 2013). The TAC is a measure of the antioxidant capacity of all antioxidants in a biological sample and not of a single compound (Nemec *et al*., 2000).

Antioxidant defense mechanisms include both enzymatic and non-enzymatic strategies. Common antioxidants include the vitamins A, C, E, and the tripeptide glutathione, and the enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase (Szaleczky *et al*., 1999).

Total antioxidant capacity has a potential to be a diabetes diagnostics and therapeutics biomarker since it has been found to be normal or increased in controlled T2D patients and reduced in uncontrolled and complicated T2D subjects (Gu *et al*., 2018).

2.6 Genetic of Retinopathy

The role of genetic factors in shaping susceptibility to diabetic retinopathy has been known for many years, family-based studies have indicated that retinopathy susceptibility is heritable, there is a high concordance of retinopathy severity among twins with both T1D and T2D (Leslie and Pyke,1982). Depending on the retinopathy phenotype and ethnic population examined, siblings and relatives of diabetic patients with retinopathy have approximately a 2- to 3-fold risk of retinopathy compared with relatives of diabetic patients without retinopathy (Arar *et al*., 2008).

Genetic factors may influence either the onset or the severity of retinopathy. In fact, heritability estimates ranging from 25% to 50% have been reported for proliferative retinopathy (Hietala *et al*., 2008).

Both hypoxia and hyperglycemia stimulate VEGF expression, and in consequence elevated VEGF and its receptor expression have been demonstrated in diabetic retinas (Simo *et al*., 2006). The VEGF gene is located in chromosome 6 (6p21.3). Many SNPs have been associated with retinopathy, most of them located in the promoter region of the gene (Vincenti *et al*., 1996). The most important one is the +405 genotype, which has been implicated in a number of diseases, in particular those with an angiogenic basis, like retinopathy (Yang *et al*., 2010). Candidate gene studies are clinical and preclinical studies where a gene is identified as potentially implicated in disease pathogenesis based on the expression of already identified proteins in the disease state, a meta-analysis study examined 34 genetic variants known to be associated with the pathogenesis of retinopathy and found the aldose reductase gene aldo-keto reductase family 1 member B have the highest number of polymorphisms associated with retinopathy irrespective of

ethnicity. Additional polymorphisms reported to be significantly associated with diabetic retinopathy included NOS3, VEGF-A, integrin subunit alpha 2, and intercellular adhesion molecule 1(Abhary *et al*., 2009).

2.6.1 Epigenetic

The detailed meaning of the term “epigenetics” that comes with a Latin prefix "epi" refers to above, over, outside, or beside genetics. Thus, it is literally referred to events that occur above and next to heredity (Tronick and Hunter, 2016). Epigenetics refer to study the phenotypic changes, which can ultimately be inherited, and which do not involve changes in the DNA sequence (Eccleston *et al*., 2007). Epigenetics including DNA methylation and histone modifications (Eggermann, 2021).

Epigenetics play a profound role in normal cellular processes, and alterations to normal epigenetic processes lead to phenotypic plasticity and disease progression, prompting widespread interest in understanding the interactions between the epigenome, the genome, and the environment (Stirzaker and Armstrong, 2021).

2.6.2 DNA Methylation

DNA methylation is the most widely studied mechanism of epigenetic field (Hernando-Herraez *et al*., 2015). It involves covalent addition of a methyl group (CH3) at the 5′ position of the cytosine ring within the 5′-CpG-3′dinucleotidesto create a 5-methylcytosine (5-mC) (Smith and Meissner, 2013). Methylation process mediated by family enzymes called DNA methyl transferases(DNMTs), with S-adenylylmethionine(SAM) that acts as a methyl donor, as shown in figure (2.1) (Miranda and Jones, 2007).

DNA methylation and histone modifications are the most well-understood epigenetic mechanisms. DNA methylation occurs on CpG dinucleotide and mainly mediates gene silencing, this mechanism involves the covalent addition of a methyl group to carbon C5 of cytosine nucleotides to create 5-methylcytosine (Wang *et*

al., 2020). The addition of methyl groups by DNA methyltransferases alters the structure of the major groove of DNA where the proteins attach, leading to heritable changes in the chromatin structure (Jones and Takai, 2001).

The methylation of the 5′- carbon of cytosine, often in a gene promoter, is a form of epigenetic modification that does affects the secondary interactions, and plays a critical role in the control of the gene expression (Hossan *et al*., 2019).

Proper DNA methylation appears to be fundamental for cell differentiation and embryonic development, whereas the aberrant methylation is associated to the disease (Zou *et al*., 2013).

Not rarely, specific methylation profiles create molecular abnormalities which cause diseases, including T2D (Jin and Liu, 2018). CpG methylation may reflect the impact of the obesity epidemic on the rise of T2D incidence (Ling and Ronn, 2019).

Recent studies in humans reported that changes in the CpG methylation pattern play a role in mediating the association between exposure to prenatal famine and increased risk of obesity, dyslipidemia, T2D and schizophrenia later in life (Tobi *et al*., 2018).

In the field of T2D, it is found that a certain SNPs related to T2D could introduce or remove CpGs (CpG-SNP), which could relate with DNA methylation and the alternative splicing events in their respective genes in pancreatic cells, indicating a conceivable role of DNA methylation in alternative splicing regulation (Dayeh *et al*., 2013).

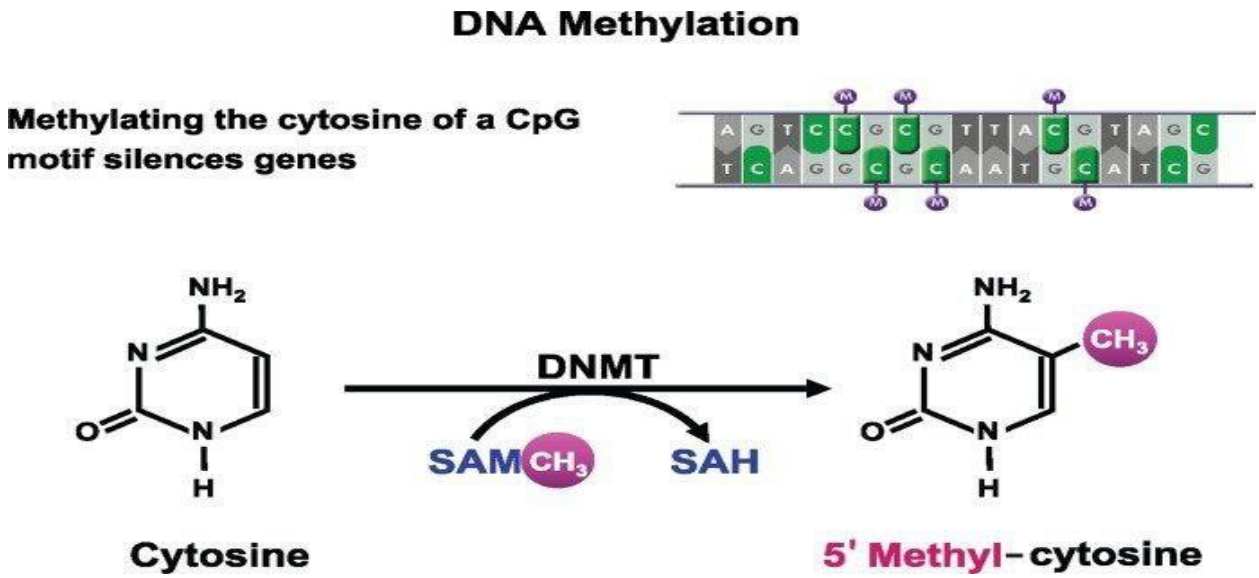

Figure (2-1) Schematic representation of DNA methylation, which converts cytosine to 5′methyl-cytosine via the actions of DNA methyltransferase (DNMT). DNA methylation typically occurs at cytosines that are followed by a guanine (i.e., CpG motifs) (Miranda and Jones, 2007).

2.6.3 Global genome methylation

Global DNA methylation refers to the average methylation status that occurs across the genome without identifying the CpG sites where they occur (Zhao *et al*., 2012).

Changes in global methylation can affect expression, genomic stability, and chromosomal structure (Jaenisch and Bird, 2003). It may play a role in the development of T2D itself and in the susceptibility to developing chronic complications through alterations of genetic expression in the vasculature of most affected tissues (Fuschi *et al*., 2019). Another manifestation of epigenetic changes in patients with diabetes is the so-called metabolic memory, whereby patients who have been poorly controlled during some time in the disease course seem to carry epigenetic changes that persist for many years and predispose them to developing complications (Intine and Sarras, 2012).

Concerning the source of the observed differences in DNA methylation, they can be attributed to different factors, but an essential role is played by

methyltransferase enzymes, especially DNA methyl transferase-1 (DNMT1),which is responsible for the maintenance of DNA methylation after cell divisions, also, disturbances of the SAM ratio may contribute to global changes in methylation, affecting the level of available SAM to perform DNA methylation (Williams and Schalinske, 2012).

Techniques used to measure global DNA methylation include polymerase chain reaction (PCR)- pyrosequencing (Bollati *et al*., 2009). Thin layer chromatography (Smolarek *et al*., 2010). Enzyme linked immunesorbent assays (ELISAs) and bisulfite sequencing, Of these, ELISA has received increased interest since it is suitable for routine analysis, it does not require sophisticated instruments and is cost-effective compared to the other methods mentioned previously (Zhao *et al*., 2012).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1 Materials

3.1.1 The Study´s Instruments and Apparatus

Table (3-1) lists the companies and places of origin of the tools and apparatuses employed in the current study.

Table (3-1): Equipments and apparatuses employed in the study

Instruments and apparatuses	Company/ Origin
Centrifuge	Back man / Germany
Deep Freeze	GFL / Germany
ELISA reader and washer	Shemadzu / Japan
Gel electrophoresis unit	Cleaver Scientific / Japan
IchromaHbA1C	Boditech Med Inc / Korea
Nano drop ND-1000	Thermo Fisher Scientific / USA
Spectrophotometer	Shemadzu / Japan
Vortex	Bioneer / Korea
Water bath	GFL / Germany
Water distillatory	GFL / Germany

3.1.2 Chemicals

Table (3-2) lists the chemical compounds used in this study along with the firms that produced them and their places of origin.

Table (3-2): Chemical Substances Used in The Study

Chemical materials	Company / Origin
Agarose	Thermo fisher / USA
Ethanol	Dissolve company/USA
Ladder 100bp size	iNtRON's Biotechnology / Korea
Loading dye	Thermo fisher/USA
TBE buffer	Bio-Basic / (England)

3.1.3 Laboratory kits

Table (3-3) provides a list of the laboratory kits that utilized in this study.

Table (3-3): Laboratory kits utilized in the study

Laboratory kits	Company / Origin
Glucose estimation kit	Linear/ Spain
Human Vascular Endothelial Cell Growth Factor -A ELISA Kit	BT LAB/ China
Ichroma HbA1C	DxGen / Korea
Insulin ELISA kit	Calbiotech Inc/ Germany
Methylamp™ DNA Modification Kit	EpiGentek Group Inc
ReliaPrep™ Blood g DNA	Promega / USA

3.1.4 Study Cases and Collection of the Blood Samples

The current study included the collection of 120 blood samples, 40 samples from healthy subjects as a control group, while another 80 samples were taken from patients with T2D, 40 one of them were diabetics with retinopathy and 40 were diabetics without any complications. They were randomly selected while attending the diabetes and endocrine care center at Marjan Hospital, and Al-Mahawil General Hospital, Babil governorate. The consent of each participant was obtained before the sampling process began. All participants were guided to fast, and all information required for the study were taken, including age, their ages ranged from 35-85 Years with disease duration from 1-24 Years, the height was also taken, weight, smoking, whether they suffer from complications or not. About 4 ml of venous blood was withdrawn in the sitting position using disposable syringes after use, 1.5 ml was placed into EDTA tubes, and the remaining 2.5 ml was placed into gel tubs to obtain serum. Blood in EDTA tubes was used to measure HbA1C, and the remaining blood was stored at 20 °C for use in DNA extraction. The gel tube, after being left for 10–15 min at room temperature, then placed in a centrifuge for 5 minutes at 2000 rpm/min; after separation, 10 µL of serum were withdrawn for use in the FBG test, and the remainder was withdrawn

and placed in Eppendorf tubes at -20 °C for use in the assessments of insulin, TAC, ROSs, and VEGF-A tests.

3.1.5 Study Design

From October 2022 until March 2023, the study was ongoing. The present investigation had conduct a case-control study to assess the importance of global DNA methylation in the development of retinopathy in patients with T2D; on the other hand, the association between methylation status and other studied parameters in progression of retinopathy in diabetes patients.

Figure (3-1) shows the design of the study, which included two aspects: the physiological study and the epigenetic study:

1. Physiological Study which include:
   A. Glycaemic control parameters (FBG, HbA1C, insulin, IR, IS)
   B. Oxidative stress parameters (TAC, ROSs, and oxidative stress index (OSI))
   C. The cytokines factor VEGF-A
2. Epigenetic study which includes:
   A. The extraction of DNA
   B. Global DNA methylation status.

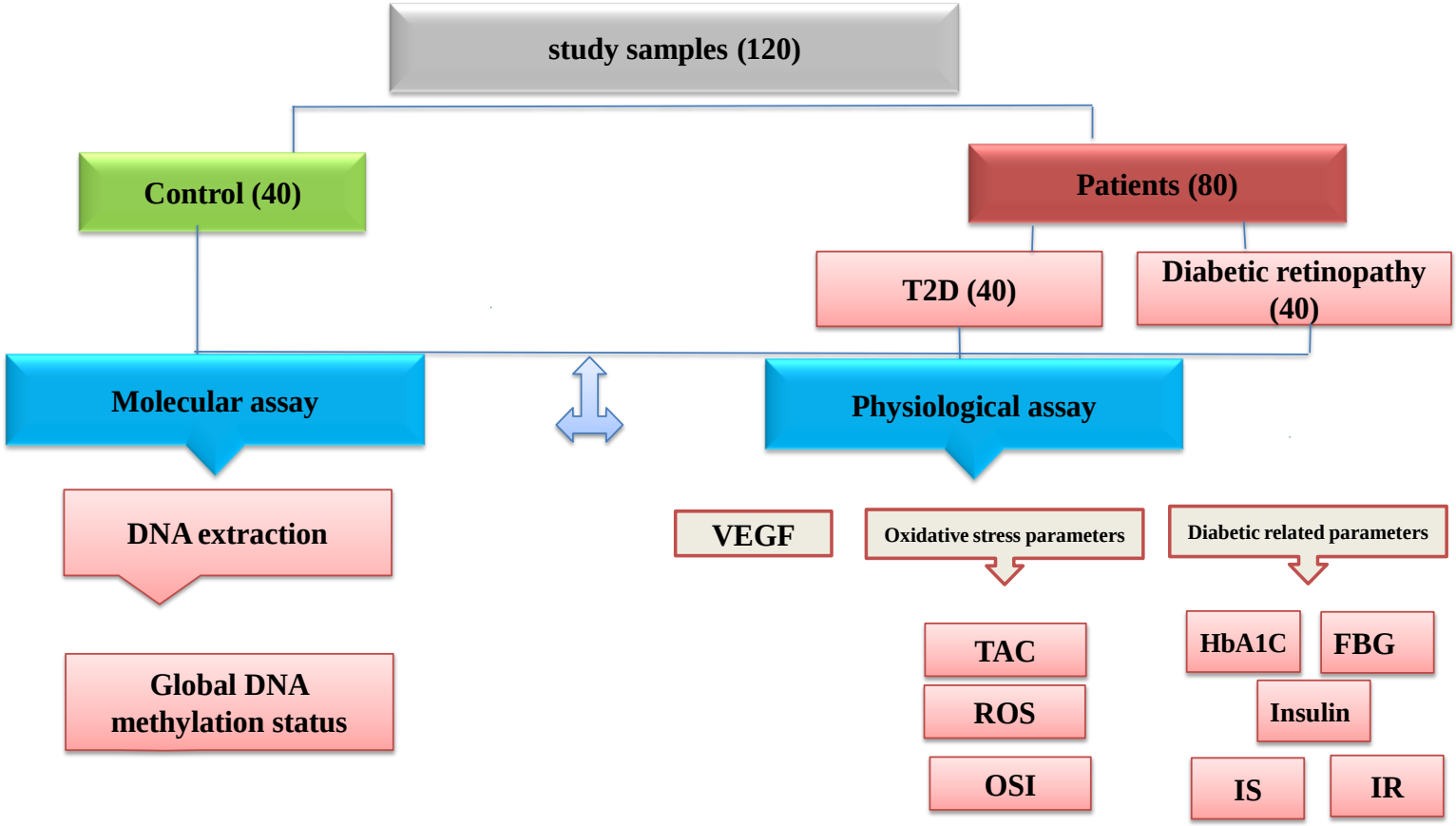

Figure (3-1): Study design

Fasting blood glucose:"FBG, Glycated hemoglobin: HbA1C, Insulin resistant: IR, Insulin sensitivity: IS, Total antioxidant capacity: TAC, Reactive oxygen species: ROS, Vascular endothelial growth factor-A: VEGF-A.

3.2 Methods

3.2.1 Determination of Body Mass Index

The BMI values of participating individuals has been calculated using the BMI formula (Jensen *et al.*, 2013):

BMI = weight (kg) / height^2 (m)^2

The weight status was classified into five groups according to the values of BMI as shown in Table (3-4).

Table(3-4): The weight status groups according to the value of BMI (European society of human reproduction and embryology, 2009).

Weight status	Values of BMI (kg/m^2)
Underweight	<18
Normal	18-24.9
Overweight	25-29.9
Obesity	30-39.9
Morbid obesity	≥ 40

3.2.2 Physiological Study

3.2.2.1 Evaluation of Fasting Blood Glucose

- **Principle**

The Linear kit, which is based on the enzymatic colorimetric method, has been used to determine serum glucose levels, in the Trinder reaction, hydrogen peroxide was created as glucose was transformed to D-gluconate by the enzyme glucose oxidase, when peroxidase is present, the combination of phenol and 4-aminoantipyrine produces a red quinoneimine dye that is directly proportional to the amount of glucose present in the sample, according to study of (Barham and Trinder, 1972).

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

$$4 - \text{aminoantipyrine} + \text{Phenol} \xrightarrow[peroxidase]{} \text{Quinoneimine} + H_2O$$

- **Examination Procedure**

1. The samples and reagents have been kept at room temperature.
2. The blanks, standards, and samples adhered to the guidelines below:

Tubes	Blank	Sample	CAL. Standard
R1. Monoreagent	1.0 mL	1.0 mL	1.0 mL
Sample	–	10 µL	–
CAL. Standard	–	–	10 µL

3. The tubes were mixed and then brought to room temperature for approximately 10 minutes.
4. In comparison to a reagent blank, the absorbance (A) of samples and standards was measured at 500 nm.

- **Calculations**

$Absorbance_{smple}/Absorbance_{standard} \times Concentration_{standard}$ = mg/dL glucose

3.2.2.2 Evalution of Glycated Hemoglobin

- **Principle**

The test employs a sandwich immune-detection approach, in which the detector antibodies in the buffer bind to antigens in the sample to create antigen-antibody complexes, which then move onto the nitrocellulose matrix and are trapped by the other immobilized antibodies on the test strip, more antigens in the sample will lead to more antigen-antibody complexes, which will result in a

stronger fluorescence signal from the detector antibodies, this stronger fluorescence signal will then be processed by the instrument for the ichroma test, which will reveal the amount of HbA1c as a percentage of total hemoglobin in the blood (Goldstein *et al*., 1995).

- **Examination Procedure**

1. The cartridge was taken from the pouch and inserted into the i-chamber.
2. About 100µl from hemolysis buffer had been transfer to the detection buffer tube.
3. After this, about 5µL of whole blood was transferred into a detection buffer tube.
4. The Cap of the insulating tube was closed and then sample mix by moving it slowly for 15 times.
5. The cartridge has been taken from the i-chamber slot.
6. About 75 µL of the sample mixture was taken by pipette and placed in a test cartridge.
7. Then, the cartridge was inserted into i-chamber slot (30° C).
8. The cartridge had left in i-chamber for about 12 minutes.
9. Finally, the test cartridge has been insert in the correct direction into the i-chroma device and read the results on the display screen of this device.

3.2.2.3 Evaluation of Insulin

- **Principle**

Insulin level in serum has been determined employing the insulin (sandwich) enzyme immunoassay kit, it is an ELISA in solid phase that is entirely founded on the sandwich concept (Kao *et al*., 1994).

- **Examination Procedure**
- First, all reagents have been allowed to equalize the room temperature (20-25ºC) and the reagents were all carefully combined use.

1. The plated strips were put into the holder in the required number.
2. About 25µl of the insulin standards, participant's serum was added into assigned wells.

3. About 100µl of the working insulin enzyme has been conjugated to the wells.
4. The microplate was mixed for about 10 seconds.
5. It was incubated for 60 minutes at room temperature.
6. The wash buffer has been used to wash the liquid three times after it has been dumped from the wells. Blot absorptive paper into the wells.
7. About 100µl of the TMB substrate has been added to all wells.
8. Incubation for about 15 minutes at the room temperature.
9. All wells have been filled with the stop solution 50µl, and the plate has been gently shaken to mix the solution.
10. The absorbance1in wells have been read on the ELISA reader at 450nm after about 15 minutes of adding the stopping solution.

The insulin concentration was calculated from the standard curve shown in Figure (3-2).

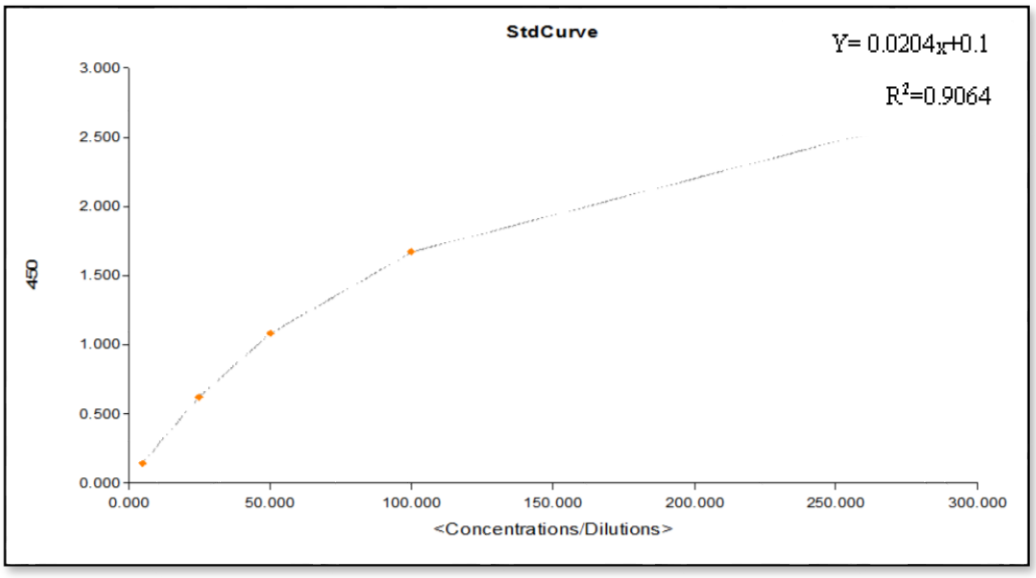

Figure (3-2): Standard Curve for Insulin Hormone

3.2.2.4 Insulin Resistance Calculation

The homeostatic-model assessment (HOMA) is a method used to quantify insulin resistance (IR) (Stumvoll and Gerich, 2001). Calculated using the equation:

HOMA-IR = Insulin * glucose / 405

3.2.2.5 Insulin Sensitivity Calculation

The levels of FBG and serum insulin concentrations for each participant are used to obtain the quantitative insulin sensitivity check index IS. It was computed using the following equation and provides a genuine, replicable, and delicate index of IS with great predictive potential (Katz *et al.*, 2000).

IS= $1/(log(fasting\ insulin\mu U/mL) + log(fasting\ glucose mg/dL)$

3.2.2.6 Evaluation of Total Antioxidant Capacity

- **Principle**

Total antioxidant capacity has been calculated using the cupric ion reduction antioxidant capacity (CUPRAC) method, which is based on an antioxidant's capacity to reduce an oxidant (Apak *et al*., 2005). $Cu2^{+}$ in the sample or standard is converted to Cu^{+}. When combined in a 2:1 complex with a chromogenic agent, this reduced copper form will selectively show up. The typical reading wavelength for this stable compound is 450 nm.

Antioxidants + Cu^{2+} ⟶ $1Cu^{+}$

$Cu^{+}1$+ 2,9-dimethyl-1,10-phenanthrolinel ⟶ complex1(ג $_{max}$ at 450 nm)

- **Examination Procedure**

The R1, R2, and the stop solution were put in the room's temperature for about 30 minutes to equalize before start working. Samples and standards have been diluted by a factor of four (1:4) in the dilution buffer given.

1. Each well has received about 200µL of diluted samples, or standard. In the absence of the sample or the standard, dilution buffer should be used as a replacement for the reagents.
2. For the purpose of reference measurement, the plate has been read at 450 nm.
3. Each well has received 50µL of Cu solution, which has been incubated for 3 minutes at room temperature.
4. The stop solution was then added in a 50µL volume.
5. At 450 nm, the plate was read a second time.

- **Calculation**

Total antioxidant capacity mmol/l $= A_{sample}/A_{standard} \times$ conc. of standard

3.2.2.7 Evaluation of Reactive Oxygen Species

- **Principle**

The ROS in serum have been evaluated using a novel method created by Erel (Erel, 2005). The ferrous ion-o-dianisidine complex is changed into ferric ion by the oxidants in the serum. The oxidation that is occurring in the reaction medium is accelerated by the glycerol molecules. In an acidic medium, ferric ions produce a colorful material that contains Xylenol orange. The intensity of the color, which is assessed using a spectrophotometer, tells us how many oxidant molecules are present in the serum. Hydrogen peroxide (H2O2) is used to calibrate the test, and the outcome is expressed in micro-molar units of H_2O_2 equivalent per liter (µmol H_2O_2Eq/l).

- **Examination Procedure**

1. The following materials were pipetted in that order:

Contents	Blank	Standard	Sample
Distilled water	50µl	--	--
Sample	--	--	50µl
Hydrogen peroxide	--	50µl	--
R1	1ml	1ml	1ml
Test tubes were mixed by vortex, and then add:			
R2	250µl	250µl	250µl

3. Following addition, the contents of each tube were gently combined; they should then be allowed to sit at room temperature for five minutes.
4. After being added, the contents of each tube were gently combined. They were then left at room temperature for 5 minutes.

- **Calculation**

Total reactived oxygen species µmol/l = $\frac{\textbf{A.sample}}{\textbf{A.standarad}}$* conc. of standard

3.2.2.8 Evaluation of Vascular Endothelial Growth Factor-A

- **Principle**

The enzyme-linked immunosorbent assay (ELISA) kit was utilized to evaluate VEGF-A. This kit contains a plate that had pre- coated with Human VEGF-A antibody. The antigens for VEGF-A in the sample is bind to antibodies coated on the wells. Then, to detect VEGF-A. The biotinylated VEGF-A Antibody is added and binds to VEGF-A in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated VEGF-A antibodies. Unbound streptavidin-HRP is washed away during a washing step, after the incubation period. After this, the substrate solution is added and color develops according the proportion of VEGF-A amount.

The reaction is stopped by adding of the stop solution and the absorbance is measured at 450 nm (Heydar *et al*., 2018).

- **Examination Procedures**

1. All reagents, reference solutions, and samples were prepared following the given instructions scrupulously. The experiment is carried out in a room temperature.
2. The strips were put in the frames to utilize them. Add 50µl standard to standard well.
3. Nearly 40µl of sample had added to the sample wells and then 10µl of anti-VEG-A antibody was added to sample wells, then 50µl streptavidin-HRP to added to sample wells and standard wells. All were mixed well and the plate was covered with asealer, then incubated 60 minutes at 37˚C.
4. To begin, the sealant had eliminated. Subsequently, the plate was cleaned for a five times with a designated wash buffer. For each of these washes, the wells had immersed in 300µl of the wash buffer for a period of 30 to 60 seconds. Before commencing the washing process with the wash buffer five times, with each well-being overfilled during automated washing, each well was crucial to be aspirate.
5. To initiate the reaction, 50µl of substrate solution A was added to each well, followed by the addition of 50µl of substrate solution B. The plate had Incubated in darkness at 37˚C for 10 minutes.
6. Each well will turn from blue to yellow when 50µl of stop solution is added.
7. Within 10 minutes after injecting the stop solution, the optical density (OD value) was read for each well using a microplate reader at 450 nm.

3.3 Epigenetic Study

3.3.1 Extraction of DNA

The genomic DNA was extracted from the whole blood samples for both T2D and control groups utilizing the ReliPrep™ Genomic DNA Miniprep system Kit (cat. No. FABGK 300).

The ReliaPrep™ Blood gDNA Miniprep System uses a simple four-step method:

1. Effectively disrupting or homogenizing the starting material to release the DNA.
2. Binding DNA to the ReliaPrep™ Binding Column.
3. Removing impurities with wash solution.
4. Eluting purified DNA. No ethanol is used in the purification protocol, eliminating downstream problems caused by ethanol carryover.

- **Examination Procedure**

The manufacturer s instructions of the kit were followed and the eluted DNA samples were stored at -20° C. These instructions were summarized as follows:

1. A blood sample was taken and mixed for 10 minutes at room temperature.
2. About 20µl proteinase k solution was added into a 1.5 ml micro-centrifuge tube.
3. About 300µl of blood was added to the tube containing proteinase k and then mixed with a vortex device.
4. About 200µl of cell lysis buffer solution was added to the content in the tube and then mixed with a vortex device for 10 seconds.
5. After mixing, it is incubated at 56˚C for 10 minutes.
6. During the incubation, the binding column was placed inside an empty collection tube according to the number of samples.
7. After taking tubes from the water bath, 250µl of binding buffer were added and then mixed with a vortex device for 10 seconds.

8. After the content appeared in green, the contents of the tube are transferred inside Reliprep binding column close it well and then putin the centrifuge for 1 minute at maximum speed.
9. The liquid is then removed as hazardous waste from the collection tube containing the flow-through.
10. The binding column was place in a new collection tube, then about 500µl of column washing solution was added and centrifuge for 3 minutes at the highest speed, the washing step was repeated for three times.
11. Then the column is placed in a 1.5ml micro-centrifuge tube,then75µl of Nuclease-Free Water was added to the column, then it is centrifuged for 1minute at the highest speed and elution the DNA.

3.3.2 DNA purity Estimation

The quality and quantity of the DNA specimen have been calculated by the Nano-drop spectrophotometry at 200 to 320nm wave length. Then, the absorbance profile has been processed and analyzed to determine the DNA quantity and quality by measuring the 260/280 and 260/230 ratios. If the DNA sample displays 260/230ratio lesser than 2 and /or 260/280 ratio lesser than 1.8, it has been re-extracted. In brief, nuclease free water (1µl) has been placed into the lower optical surface lever arm and selected for the application software which was measured on 260/280 nm wave length, and the concentration of DNA was automatically counted up by software that offered on the personal computer screen which was linked to the Nano-drop.

3.3.3 DNA Integrity Estimation

Integrity of extracted DNA has been detected by the agarose gel electrophoresis. It was done according to Samboork and Russell (2001) as summarized below:

1. The comb has been putted in their position on gel casting tray to form appropriate wells. The open ends of the casting tray were blocked with a

moveable gates way, and the grinder of the comb were placed about 0.5 mm over gel bottom.

2. In order to create 500ml of Tris Borate EDTA buffer (TBE) (1X), 50ml of TBE(10X) stock solution was added to 500ml of deionized water.
3. The agarose gel (1%) has been prepared by resolving 0.4g of agarose in 40ml of TBE (1X) and it was heated by a hot stirrer plate until it melted.
4. About 1µl of ethidium bromide has been added to the agarose gel merely before pouring it into the tray and blending swirling.
5. Agarose gel has been poured into the casting tray and allowed it to be solid for about 25 min. Once the gel solidified, the comb was removed with a mild lower back and forth movement while taking care to not rip up the gel. Then the quit gates were lowered.
6. The hardened gel has been transferred to the electrophoresis instrument and immersed with TBE (0.5X) buffer until it was reached a level of about 0.5-1 cm above gel surface.
7. First, 3µl of ladder marker has been added to the gel, then 6µl of each extracted DNA was mixed with 4µl of the loading dye and added it was in the other wells.
8. When the lid has been placed on the gel box, and the electrodes have been connected, and turned on the power supply. The DNA would be travel towards the positive (red) electrode away from the well.
9. Electrophoresis has been executed by setting the device on 75 volts until the tracking dye moved at least 10 cm of the gel length.

3.3.4 Photo Documentation

The agarose gel has been visualized in the UV trans illuminator that used to be supplied with the gel documenting unit, and the agarose gel has been positioned above the UV trans illuminator device, and it was exposed to UV mild and the pictures were picked up utilizing Canon digital.

3.3.5 Evaluation of Global DNA Methylation

- **Principle and Procedures**

1. The Methyl Flash™Methylated DNA Quantification Kit (Colorimetric) contains all the necessary reagents necessary to detect the global DNA methylation as the examination steps begin: Genomic DNA preparation (DNA isolation was performed using the Quick-g DNA™ Blood MiniPrep).
2. Binding the DNA to the assay's wells (48 or 96 well plate).
3. Following the binding of genomic DNA to assay's Wash the wells, and then add the antibody to detect the methylated fraction of DNA.
4. Wash wells, and then add detection antibody and enhancer solution.
5. Add color developing solution for color development, then measure absorbance in the microplate spectrophotometer where the amount of methyl DNA is proportional to the measured OD intensity.

- **5mC Calculation**

To determine the relative methylation status of two different DNA samples, a simple calculation of the 5-mC percentage in total DNA is performed using the following formula:

$$5\text{-}mC\% = \frac{(\text{SampleOD-ME3OD}) \div S}{(\text{ME4OD-ME3OD}) \text{x} 2^{*} \div P} \; x100\%$$

S is the amount of input sample DNA in ng.

P is the amount of input positive control (ME4) in ng.

*2 is a factor to normalize 5-mC in the positive control to 100%, as the positive control contains only 50%of 5-mC.

$$5-mC(ng)=\frac{Sample\ OD\ -\ ME3\ OD}{Slope\ x\ 2\ *}$$

$$5-mC\%=\frac{5-mC\ Amount\ (ng)}{S}\times 100\%$$

S is the amount of input sample DNA in ng.

*2 is a factor to normalize 5-mC in the positive control to 100%, as the positive control contains only 50% of 5-mC.

3.4 Statistical Analysis

Current research data were analyzed using statistical package of social science (SPSS)version 26. The data were expressed as mean (± SD). Statistical comparisons between groups were made applying T-test and a P value of ≤ 0.05 was considered significant using an analysis of variance (Anova). Moreover, Pearson correlation coefficients and regression analysis were computed to examine the association between the factors under study (Dunken *et al.,* 1983).

Chapter Four

The Results

4. Results

The results of present study included:

1. Physiological study
2. Epigenetic study

The study included a physiological assessment glyceamic control parameters (FBG, HbA1C, Insulin, IR, IS); oxidative stress parameters (TAC, ROS, OSI); and the cytokine VEGF-A. The epigenetic study involved the assessment of global DNA methylation in all studied groups.

4.1 Descriptive Data of Study Groups

Table (4-1) shows some important characteristics for both diabetics and healthy control subjects such as age, BMI, gender and smoking status. As the table shows, there are a significantly($p \leq 0.05$) increase in the mean of age (56.38±10.39 VS. 39.125± 9.72) and BMI (30.75±5.8 VS. 27.54± 4.27) for T2D patients compared with healthy control subjects.

As for gender, identical numbers of males and females were taken for both patients and healthy subjects. On the other hand, the current study record non-significant ($P>0.05$)differences in study groups according to smoker habit (patients 41% VS. 42.5% in control).

Table (4-1): The Descriptive data of study groups (healthy control and diabetic patients)

Variables	Mean ± SD		Odds ratio	95%CI	P-value
	Healthy Control	Diabetic patients			
Age (years)	39.125± 9.72	56.38±10.39	t =2.225		0.00*
BMI (kg/m2)	27.54± 4.27	30.75±5.8	t =2.1		0.00*
Gender					
(Male/Female)	40 (20/20)	80 (40/40)			
Smoking					
Smoker	17 (42.5%)	33 (41.25%)	OR=1.05	0.6-1.85	0.32^{NS}
Non smoker	23 (57.5%)	47 (58.75)			

SD: Standard Deviation, CI: Confidence interval,

Significant*($p \leq 0.05$), NS: Non Significant

4.2 Physiological Study

4.2.1 Assessment of glyceamic control and oxidative stress parameters in Studied Population

Table (4-2) shows the assessment of related glyceamic control parameters(FBG, HbA1C, Insulin, IR, IS) and table (4-3) shows the assessment of related oxidative stress parameters (TAC, ROS, OSI); whereas figures (4-1), (4-2) display levels of VEGF-A in all study population. This assessment includes between groups comparison (all diabetic patients and control) and within group comparison (diabetic retinopathy and T2D without complication).

As indicated in this table there are a highly significant increased ($P \leq 0.05$)in FBG, HbA1C, insulin and IR in T2D patients as compared with healthy control,

whilst IS had a significant ($p \leq 0.05$) increase in healthy control compared to T2D patients. On the other hand, within group comparison shows increased values of most glyceamic control parameters in retinopathy patients, but the results of statistical analysis using T-test showed that only FBG and insulin have a significant ($p \leq 0.05$) differences.

As for oxidative stress markers, the results of statistical analysis demonstrated a highly significantly ($P \leq 0.001$) raise in TAC and, asignificant($P \leq 0.05$) increase in ROS and OSI in total diabetic patients compared with control. While the comparison within patients group demonstrated non-significant ($P > 0.05$) differences in TAC, a significant ($P \leq 0.05$) increase in levels of ROSs and OSI were shown in retinopathy patients compared with T2D without complications. The assessment of VEGF-A levels in studied groups displayed a significant ($P \leq 0.05$) boost in diabetic patients (115.5±35.5) as compared with control (49.3±23.02). Also, within group comparison indicated a significant($P \leq 0.05$) raised in the concentration of VEGF-A in retinopathy (128.35±22.11)compared with (100.93±23.31) in T2D patients, P=0.022*, as shown in Figures (4-2).

Table (4-2): Assessment of glyceamic control parameters in diabetic patients and healthy control subjects

Parameters	Mean ± SD			
	Healthy controls	Total patients	Diabetic retinopathy	T2D
FBG (mg/dl)	85.63±16.29	199±72.48	231.98±74.37	202.72±75.86
P value	0.00**		0.05*	
HbA1C (%)	5.01±0.57	9.02±3.01	9.05±1.88	9.19±2.16
P value	0.00**		0.31^{NS}	
Insulin (µIU/ml)	6.26±2.31	16.27±6.02	24.10±9.27	19.82±8.01
P value	0.00**		0.02*	
IR	1.31±0.18	8.12±3.01	13.13±6.17	11.62±7.34
P value	0.00**		0.20^{NS}	
IS	0.39±0.01	0.31±0.1	0.27±0.02	0.28±0.02
P value	0.00**		0.81^{NS}	

SD: Standard Deviation, NS: Non-Significant, NS (P>0.05).*(P≤0.05) , **(P<0.001)

Table (4-3): Assessment of Oxidative stress parameters in diabetic patients and healthy control subjects

Parameters	Mean ± SD			
	Healthy controls	Total patients	Diabetic retinopathy	T2D
TAC (mmol/l)	790.88±146.31	1001.05±215.69	971.92±202.44	1033.15±230.2519
P value	0.00**		0.13^{NS}	
ROS (µmol/l)	15.59±7.78	28.70±8.99	33.02±12.5	24.51±10.87
P value	0.003**		0.021*	
OSI %	1.28±0.66	3.07±1.92	3.50±2.10	2.44±1.57
P value	0.046*		0.018*	

SD: Standard Deviation, NS: Non-Significant, NS (P>0.05).*(P≤0.05) , **(P<0.001)

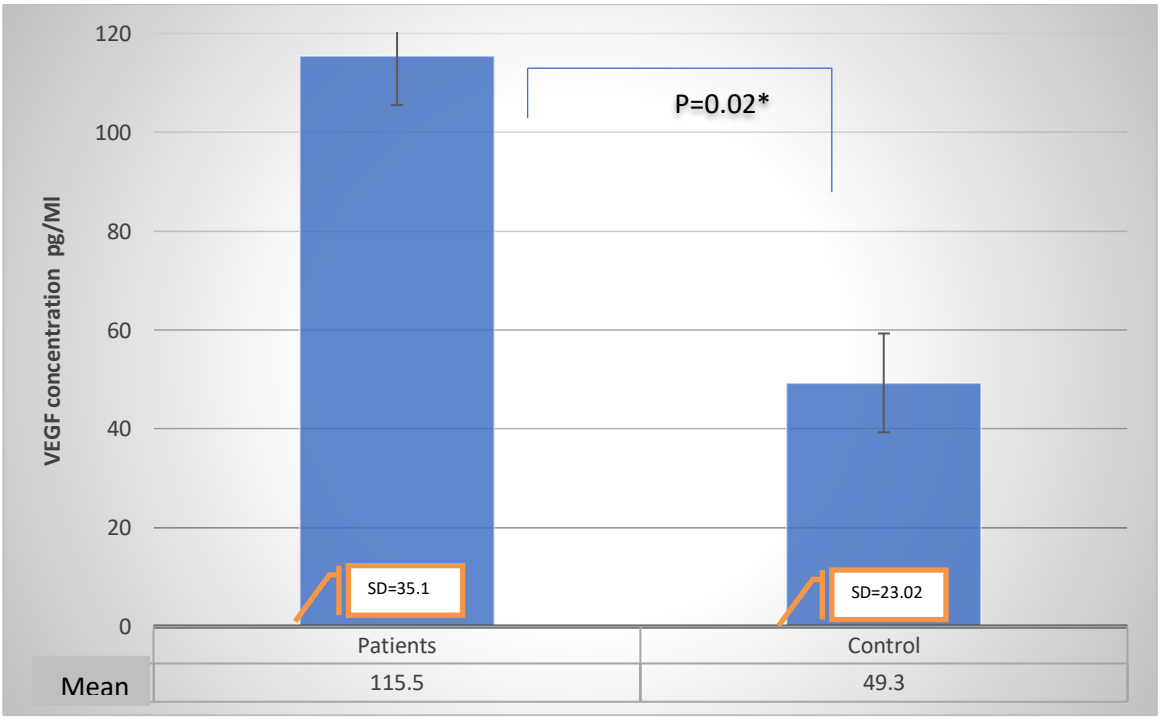

Figure (4-1): Assessment of VEGF-A in diabetic patients and healthy controls subject.

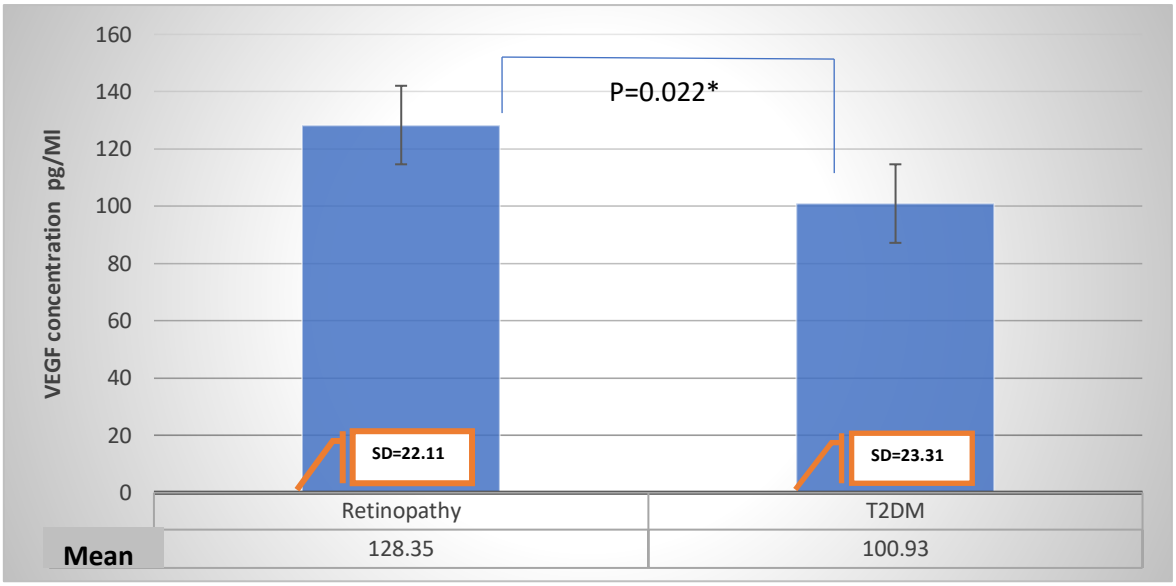

Figure (4-2): Comparison of VEGF-A in retinopathy and T2D patients

4.2.2 Distribution of diabetic patients according to age

Distribution of the retinopathy and T2D patients according to their age is elucidated in figures (4-3) and (4-4). They were divided into four age categories: (35-44, 45-54, 55-64 and≥65 years), The highest percentage for retinopathy and T2D patients were within age categories 55-64 and ≥65 years respectively, while the lowest percentage were within age categories 35-44 and 45-54 years.

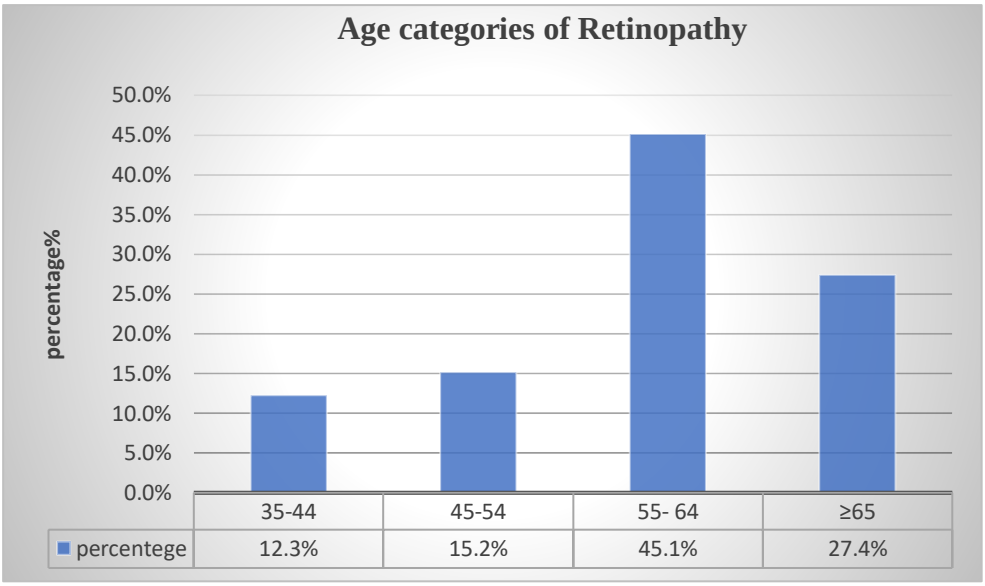

Figure (4-3): Distribution of retinopathy patients according to the age

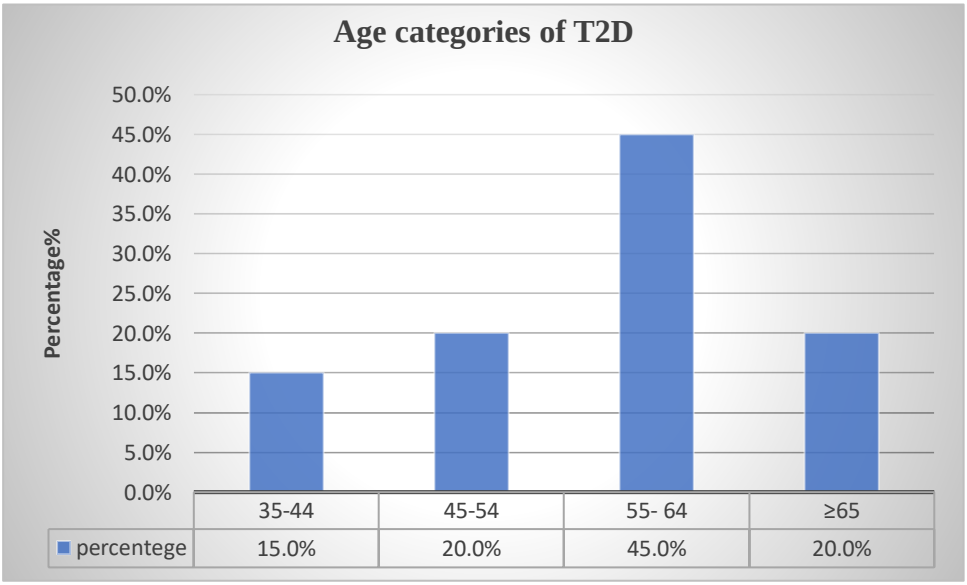

Figure (4-4): Distribution of T2D patients according to the age

4.2.3 Impact of Age on the glyceamic control and Oxidative stress Parameters in Both Diabetic Retinopathy and T2D Patients

Table (4-4) illustrates the effect of age on levels of the glyceamic control parameters in both diabetic retinopathy and T2D. The results of the statistical analysis using one-way ANOVA showed that there was asignificant difference for the physiological markers that distributed according to age in both patients with

diabetes and retinopathy. In both groups (diabetic retinopathy and T2D), the current study found that FBG have a significant ($p \leq 0.05$) increase in55-64 and≥65age groups compared with others. While HbA1C, insulin and IR showed a significant ($p \leq 0.05$) increase in≥65age group compared with others. IS showed in-significant ($p > 0.05$) differences among age categories .

Table (4-5) illustrates the effect of age on levels of the oxidative stress parameters where she indicated, the findings indicated that TAC had a non-significant ($p > 0.05$) differences among age categories. Whereas ROS and OSI have a significant ($p \leq 0.05$)differences within age categories, the highest levels were in ≥65 for retinopathy, while the highest levels were in 55-64 and≥65 for T2D patients.

The distribution of VEGF-A concentrations according to age groups is shown in the figures (4-5) and (4-6) for retinopathy and T2D, respectively. The levels of VEGF-A showed a significant ($p \leq 0.05$)increase in≥65age group in both diabetic retinopathy and T2D.

Table (4-4): Impact of Age on glyceamic control parameters in T2D and retinopathy patients

The glyceamic control Parameters						
Group	**Age (year) categories**	**FBG (mg/dl)**	**HbA1C%**	**Insulin (µIU/ml)**	**IR**	**IS**
Retinopathy	**35-44**	154.9±10.54^{*b}	7.8455±0.35^{*c}	19.4±2.64^{*b}	7.46±1.16^{*c}	0.28±0.01^{a}
	45-54	166.8±88.53^{*b}	8.262±1.10^{**c}	23.8±8.4^{*b}	13.15±4.22^{*b}	0.27±0.03^{a}
	55-64	210.5±88.01^{*a}	8.9655±1.80^{*b}	22.4±5.59^{*b}	13.26±8.10^{*b}	0.27±0.02^{a}
	≥ 65	228.2±79.31^{*a}	10.02±0.49^{*a}	26.35±13.97^{*a}	15.94±8.95^{*a}	0.26±0.01^{a}
P value		0.011*	0.016*	0.046*	0.054*	0.170^{NS}
T2D	**35-44**	187.2±50.27^{*b}	8.01±1.78^{*d}	18.22±8.09^{*c}	8.14±5.91^{*d}	0.29±0.01^{a}
	45-54	201.37±72.20^{*b}	8.97±1.62^{*c}	17.16±7.50^{*c}	10.67±6.62^{*c}	0.28±0.02^{a}
	55-64	253.41±60.43^{*a}	9.24±2.22^{*b}	20.61±9.20^{*b}	12.74±8.52^{*b}	0.27±0.02^{a}
	≥ 65	245.23±24.39^{*a}	10.44±2.51^{*a}	25.83±16.4^{*a}6	15.66±9.79^{*a}	0.25±0.02^{a}
P value		0.013*	0.020*	0.051*	0.038*	0.066^{NS}

Different small Letter refer to significant among groups comparison, similar Letters refer to non-significant differences, SD: Standard Deviation, NS: Non-Significant. *(P≤0.05), NS (P>0.05)

Table (4-5):Impact of Age on Oxidative stress parameters in T2D and retinopathy patients

Oxidative Stress Parameters				
Group	**Age(year) categories**	**TAC (mmol/l)**	**ROS (µmol/l)**	**OSI %**
Retinopathy	35-44	823.87±132.61 [a]	60.12±33.69[*b]	8.03±5.29[*a]
	45-54	1036.93±199.56 [a]	55.74±37.58[*b]	5.32±3.15[*b]
	55-64	958.53±246.89 [a]	67.18±36.48[*b]	7.67±4.84[*a]
	≥ 65	1133.96±211.47 [a]	91.41±39.60[*a]	8.61±3.25[*a]
P value		0.12[NS]	0.00*	0.00*
T2D	35-44	1123.32±396.54 [a]	31.043±21.73[*c]	2.77±1.44[*b]
	45-54	1053.21±172.69 [a]	53.07±43.60[*b]	6.51±3.21[*a]
	55-64	981.12±183.15 [a]	74.82±41.42[*a]	5.32±3.99[*a]
	≥ 65	1162.78±119.88 [a]	86.92±39.17[*a]	7.45±3.35[*a]
P value		0.12[NS]	0.00*	0.00*

Different small Letter refer to significant among groups comparison, similar Letters refer to non-significant differences, SD: Standard Deviation, NS: Non-Significant. [*](P≤0.05), NS (P>0.05)

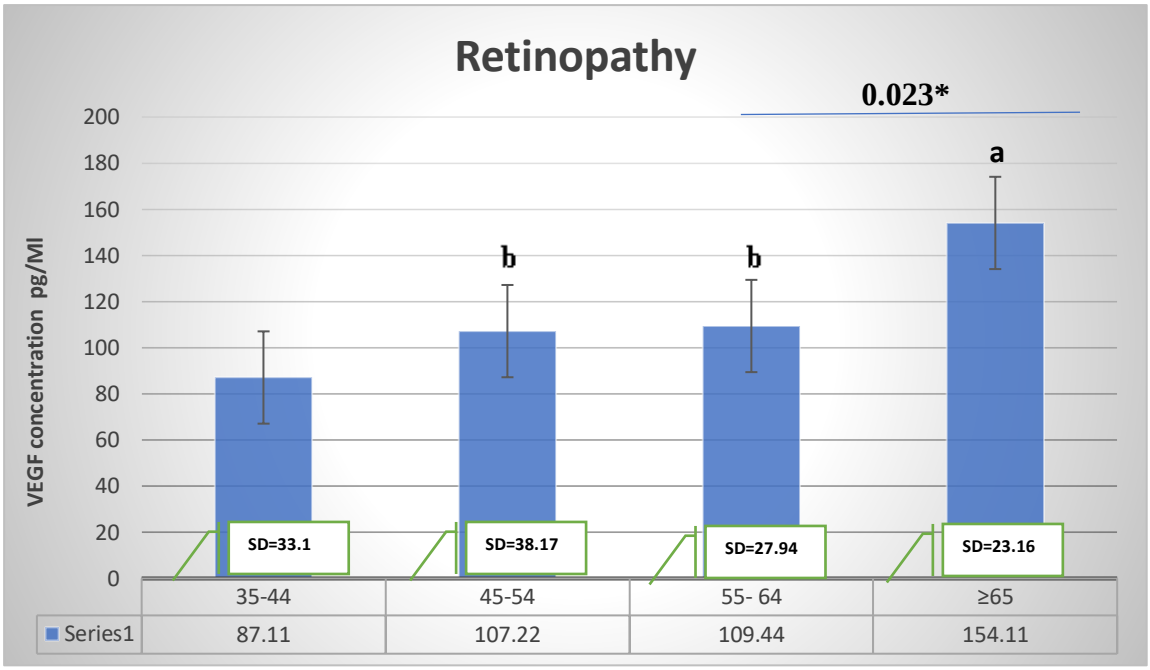

Figure (4-5): Impact of age on VEGF-A concentration in retinopathy patients

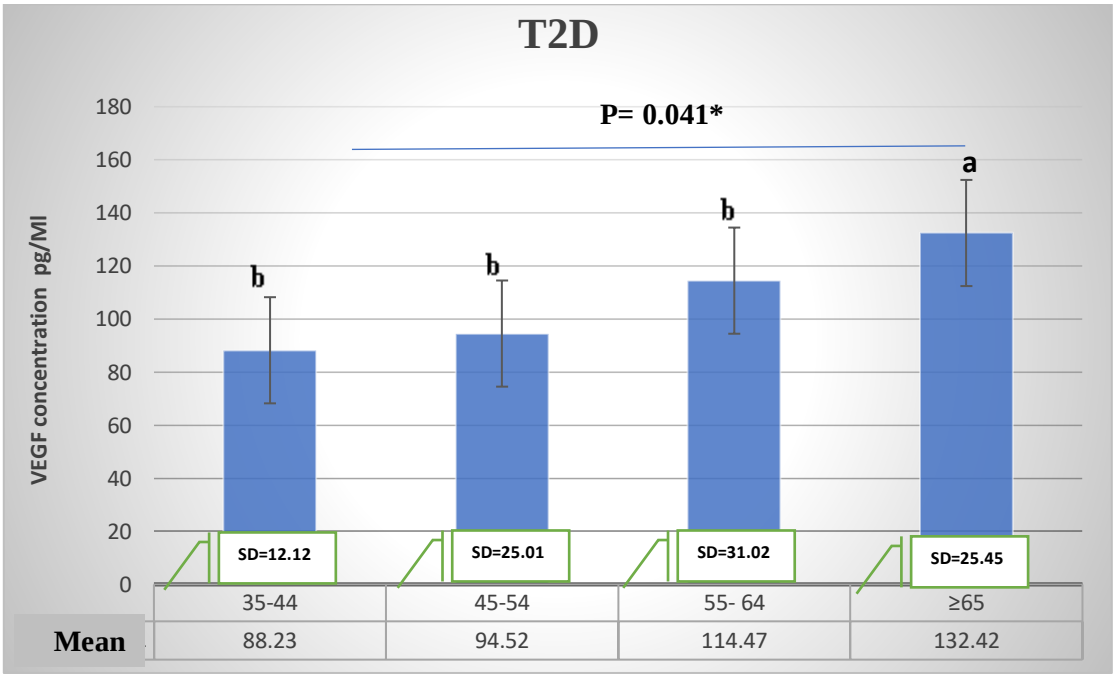

Figure (4-6): Impact of age on VEGF-A concentration in T2D patients

4.1.4 Distribution of Diabetic Patients According to BMI

Distribution of the retinopathy and T2D patients according to their BMI values is elucidated in figures (4-7) and (4-8). They were divided into four BMI categories: (Normal, Overweight, Obesity and Over obesity), The highest percent for retinopathy and T2D patients were within obesity group.

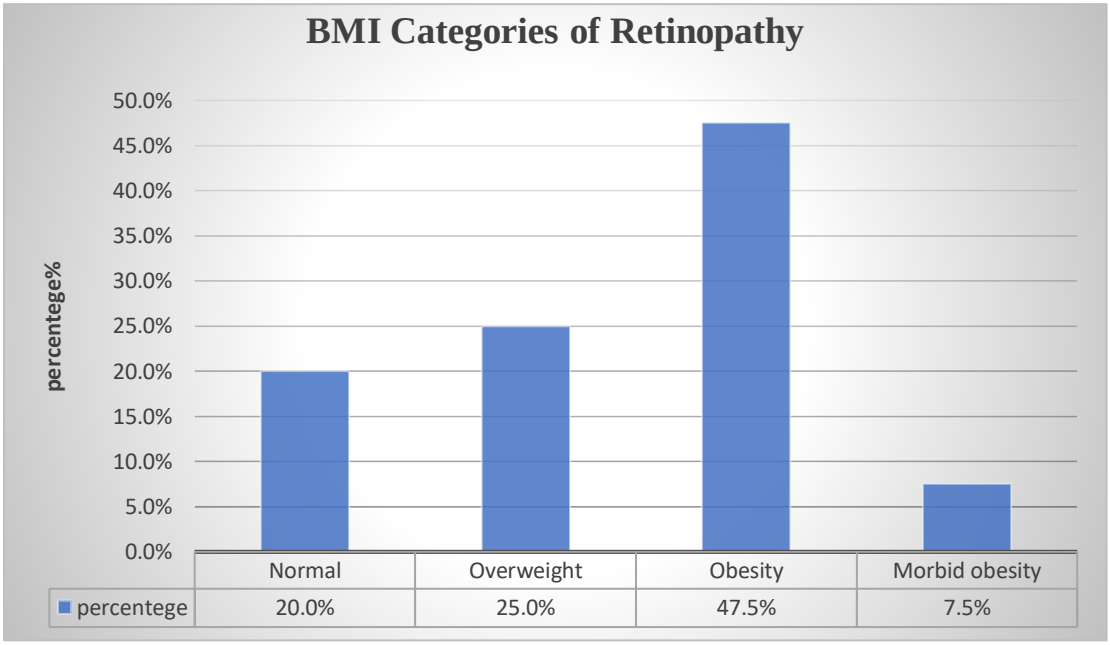

Figure (4-7): Distribution of retinopathy patients according to the BMI (kg/m2)

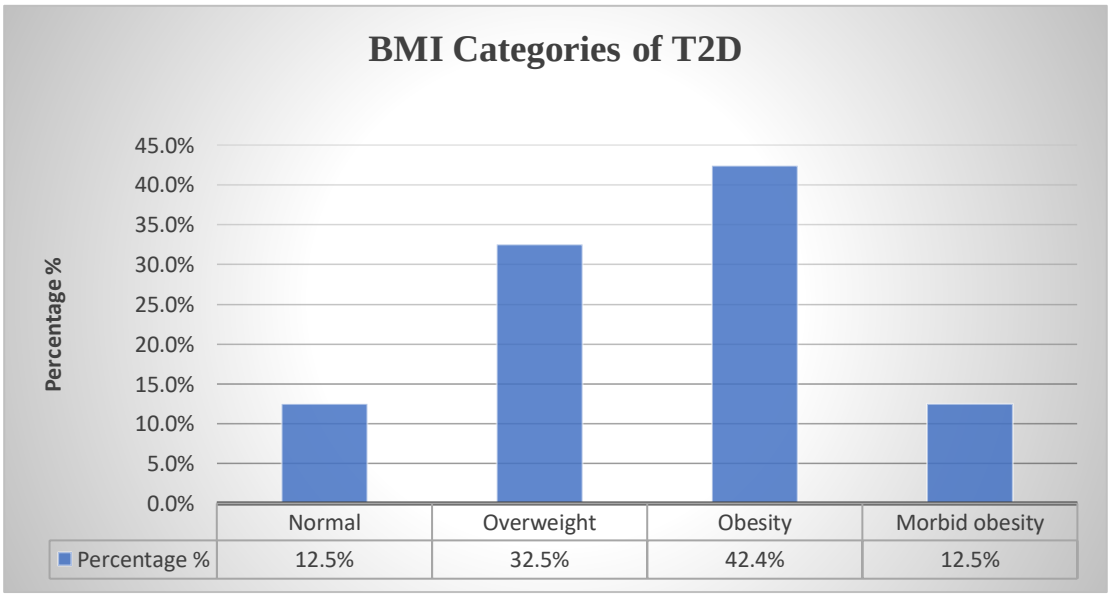

Figure (4-8): Distribution of T2D patients according to the BMI (kg/m2)

4.1.4.1 Impact of BMI on the glyceamic control parameters and Oxidative stress parameters in Both Diabetic retinopathy and T2D Patients

The effect of BMI on glyceamic control parameters in retinopathy and T2D is explained in table (4-6). The statistical analysis using one-direction ANOVA was employed to detection the considerable differences in the levels of both diabetic related parameters and oxidative stress and VEGF-A factor. table (4-6) indicated a considerable ($P \leq 0.05$) effect in the levels FBG among BMI groups of all patients, the highest levels of FBG were in obesity and morbid obesity in both retinopathy and T2D. A considerable ($P \leq 0.05$) increased also was found in HbA1C level in morbid obesity compared with other BMI groups in T2D only. Also, a significant ($P \leq 0.05$) increased was found in the levels of insulin and IR in obesity and morbid obesity in both retinopathy and T2D. In the other hand, the value of IS showed significantly ($P \leq 0.05$) decreased in morbid obesity group compared with other BMI groups.

Table(4-7) show the effect of BMI on oxidative stress Index parameters in retinopathy and T2D, present study recorded asignificant ($P \leq 0.05$) increased in TAC in normal weight and overweight groups in retinopathy; and asignificant ($P \leq 0.05$) increased within normal weight group only in T2D. The highest levels of ROS and OSI were in obesity and morbid obesity in both retinopathy and T2D, with significant ($P \leq 0.05$) differences.

According to VEGF-A factors, a significant ($P \leq 0.05$) increased was found in obesity and morbid obesity groups compared with other BMI groups in both retinopathy and T2D patients as shown in figures (4-9) and (4-10), respectively.

Table (4-6): Impact of BMI on glyceamic control parameters in retinopathy and T2D patients

The Glyceamic control Parameters						
Group	**BMI categories**	**FBG (mg/dl)**	**HbA1c%**	**Insulin (µIU/ml)**	**IR**	**IS**
Retinopathy	**Normal**	180.51±58.87	8.29±1.79 ^{a}	17.16±2.5^{*b}	9.15±4.8^{*b}	0.28±0.02^{*a}
	Overweight	202.91±65.73^{*b}	8.79±1.48 ^{a}	20.83±5.71^{*b}	11.02±5.3^{*b}	0.27±0.02^{*b}
	Obesity	278.52±72.13 *^{a}	9.31±2.18 ^{a}	28.66±9.37 ^{a*}	21.11±13.5^{*a}	0.25±0.01^{*d}
	Morbid obesity	254.01±65.55*^{a}	8.99±2.12^{a}	26.1±8.96 ^{a*}	18.53±7.89^{*a}	0.26±0.04^{*c}
P value		0.01*	0.13^{NS}	0.03*	0.01*	0.01*
T2D	**Normal**	169.22±70.48^{*c}	8.39±1.06^{*c}	18.4±8.47^{*b}	9.56±5.20^{*c}	0.28±0.01^{*a}
	Overweight	206.88±74.94^{*b}	8.61±1.5^{*c}	20.46±8.25^{*b}	10.99±8.83^{*c}	0.28±0.03^{*a}
	Obesity	225.12±83.79^{*a}	9.59±2.02^{*b}	25.53±11.07^{*a}	13.81±7.54^{*b}	0.26±0.02^{*b}
	Morbid obesity	255.15±28.19^{*a}	11.77±1.75^{*a}	30.2±8.29^{*a}	19.65±4.21^{*a}	0.25±0.007^{*c}
P value		0.00**	0.020*	0.004**	0.001**	0.01*

Different small Letter refer to significant among groups comparison, similar Letters refer to non-significant differences, SD: Standard Deviation, NS: Non-Significant. *(P≤0.05), NS (P>0.05).

Table (4-7): Impact of BMI on Oxidative stress parameters in retinopathy and T2D patients

Oxidative Stress parameters				
Group	**BMI categories**	**TAC (mmol/l)**	**ROS (µmol/l)**	**OSI %**
Retinopathy	**Normal**	1227.4±149.68 [a]	18.62±4.12 [*c]	2.01±1.07 [*b]
	Overweight	1036.2±198 [a]	26.9±6.62 [*b]	2.51±0.7 [*b]
	Obesity	799.78±105.79 [*b]	34.62±9.89 [*a]	3.77±2.08 [*a]
	Morbid obesity	879.87±160.35 [*b]	37.97±13.32 [*a]	3.92±1.04 [*a]
P value		0.01*	0.006*	0.006**
T2D	**Normal**	1284.51±197.12 [*a]	19.89±3.54	1.45±0.25 [*b]
	Overweight	979.91±196.43 [*b]	22.6±10.54 [*b]	3.14±1.15 [*a]
	Obesity	918.51±195.78 [*b]	29.48±14.75 [*a]	3.43±1.10 [*a]
	Morbid obesity	771.06±178.77 [*b]	31.99±14.2 [*a]	3.78±2.12 [*a]
P value		0.04*	0.001**	0.02*

Different small Letter refer to significant among groups comparison, similar Letters refer to non-significant differences, SD: Standard Deviation, NS: Non-Significant. *(P≤0.05), NS (P>0.05).

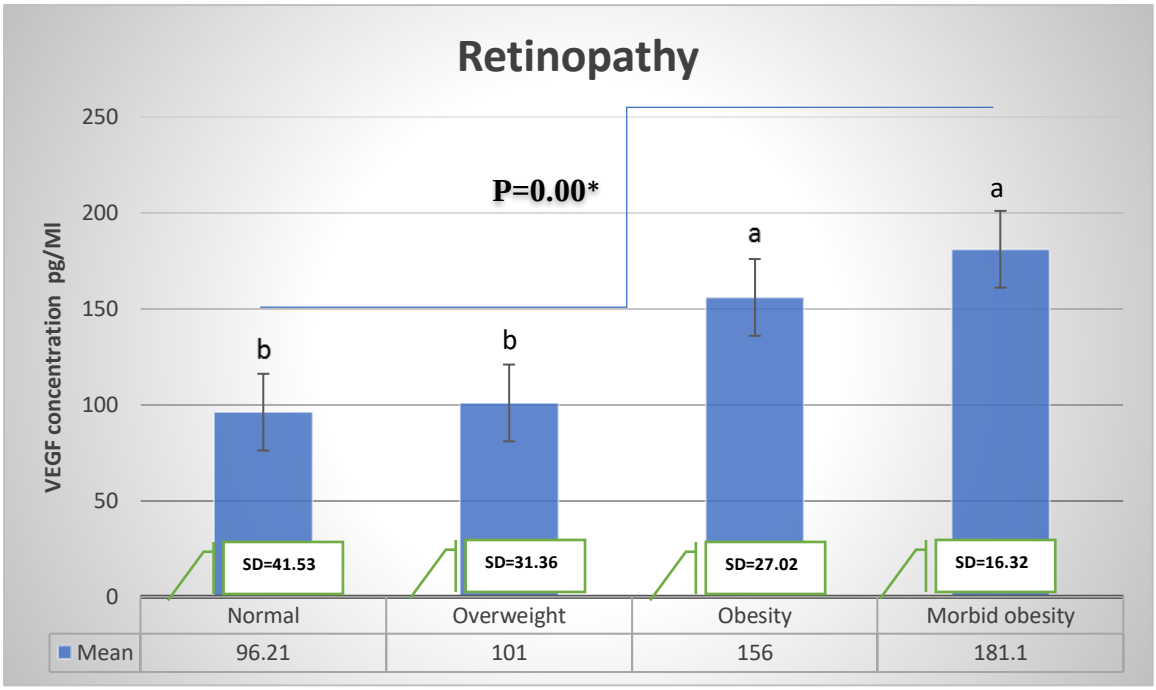

Figure (4-9): Impact of BMI on VEGF-A concentration in Retinopathy patients

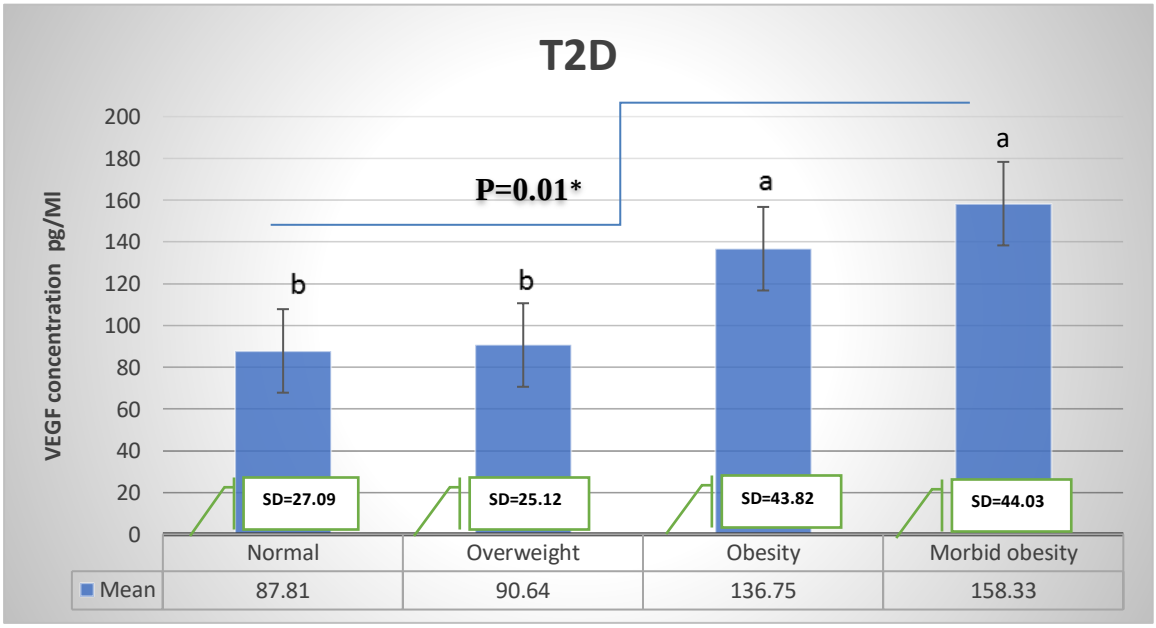

Figure (4-10): Impact of BMI on VEGF-A concentration in T2D patients

4.1.5 Distribution of Diabetic Patients According to Gender

Males and females were distributed equally in all study groups, as shown in the figure (4-11).

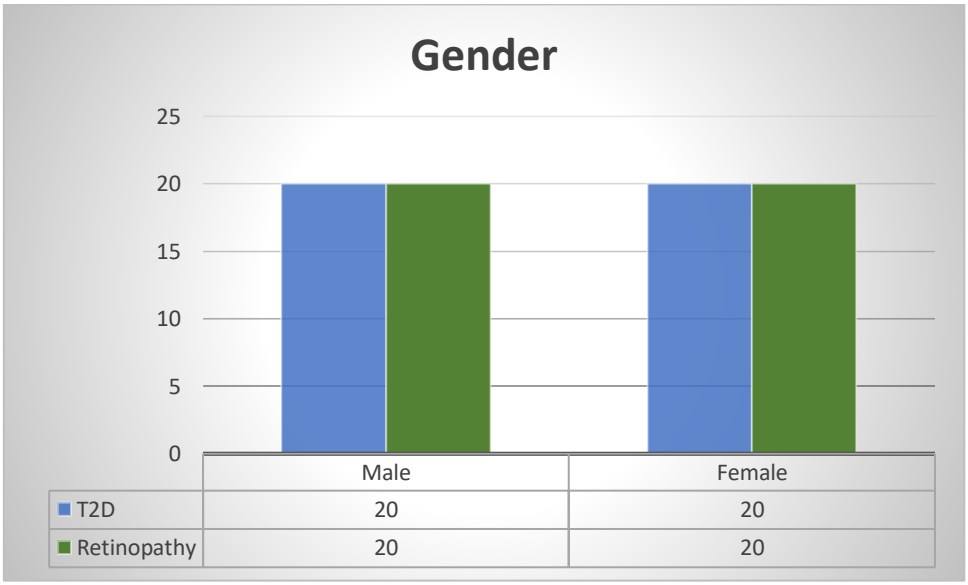

Figure (4-11): Distribution of study population according to the Gender

4.1.5.1 Impact of Gender on the glyceamic control and oxidative stress parameters in Both Diabetic Retinopathy and T2D Patients

The effect of gender on glyceamic control parameters and effect of gender on oxidative stress index in both Diabetic retinopathy and T2D patients were elucidated in Table (4-8), Table(4-9) Figure (4-12) and Figure(4-13). The statistical analysis using T- test revealed a significiant ($P \leq 0.05$) increased in levels of HbA1C in female compared with male in retinopathy group only, while other diabetic related parameters showed non-significant differences ($P > 0.05$) in both groups. Regarding the oxidative stress markers, the statistical analyses showed a considerable ($P \leq 0.05$) increased in TAC value in males while ROS and OSI showed asignificant ($P \leq 0.05$) in females compared with male in T2D only. In retinopathy patients TAC, ROS and OSI showed non-significant differences ($P > 0.05$) between male and female. As for the VEGF factor, the results of the present study showed a significant ($P \leq 0.05$) increased in females compared with males only in retinopathy.

Table (4-8):Impact of Gender on glyceamic control parameters in retinopathy and T2D patients

The glyceamic control Parameters						
Group	**Gender**	**FBG (mg/dl)**	**HbA1c%**	**Insulin (µIU/ml)**	**IR**	**IS**
Retinopathy	**Females**	244.89±82.91	10.16±2.34	21.75±9.73	13.05±5.59	0.27±0.02
	Males	219.04±62.02	8.73±1.62	17.7±6.07	9.84± 4.65	0.28±0.02
P value		0.14^{NS}	0.00**	0.11^{NS}	0.14^{NS}	0.29^{NS}
T2D	**Females**	216.03±67.61	9.06±2.09	25.05±8.34	13.38 ±5.2	0.27±0.02
	Males	200.98±77.99	8.85±1.17	22.95±6.21	12.74±3.93	0.27±0.02
P value		0.21^{NS}	0.37^{NS}	0.29^{NS}	0.42^{NS}	0.33^{NS}

Different small Letter refer to significant among groups comparison, similar Letters refer to non-significant differences,

SD: Standard Deviation,

NS: Non-Significant. *($P \leq 0.05$), NS ($P > 0.05$)

Table (4-9): Impact of Gender on Oxidative stress parameters in retinopathy and T2D patients

Oxidative Stress Parameters				
Group	**Gender**	**TAC (mmol/l)**	**ROS (µmol/l)**	**OSI %**
Retinopathy	**Females**	890.69±241.20	30.10±5.76	3.06±1.79
	Males	1008.57±219.70	32.87±8.41	3.78±2.18
P value		0.08^{NS}	0.35^{NS}	0.15^{NS}
T2D	**Females**	845.52±196.37	29.47±7.30	2.97±1.88
	Males	1098.65±208.72	19.87±8.4	1.61±0.8
P value		0.01*	0.02*	0.011*

Different small Letter refer to significant among groups comparison, similar Letters refer to non-significant differences, SD: Standard Deviation, NS: Non-Significant. *(P≤0.05), NS (P>0.05)

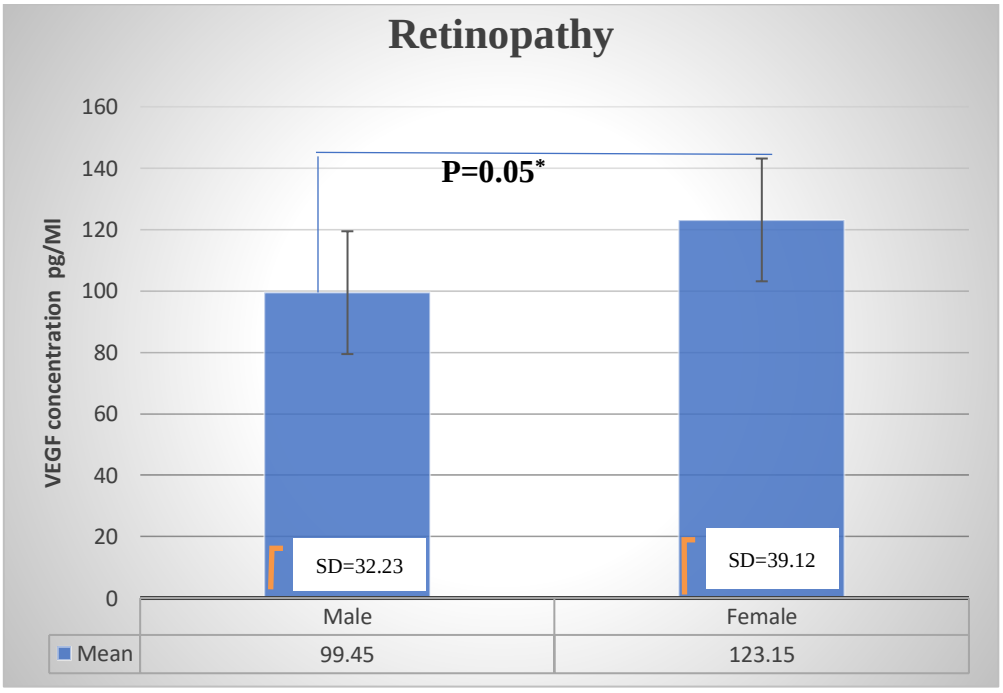

Figure (4-12): Impact of Gender on VEGF-A concentration in Retinopathy patients

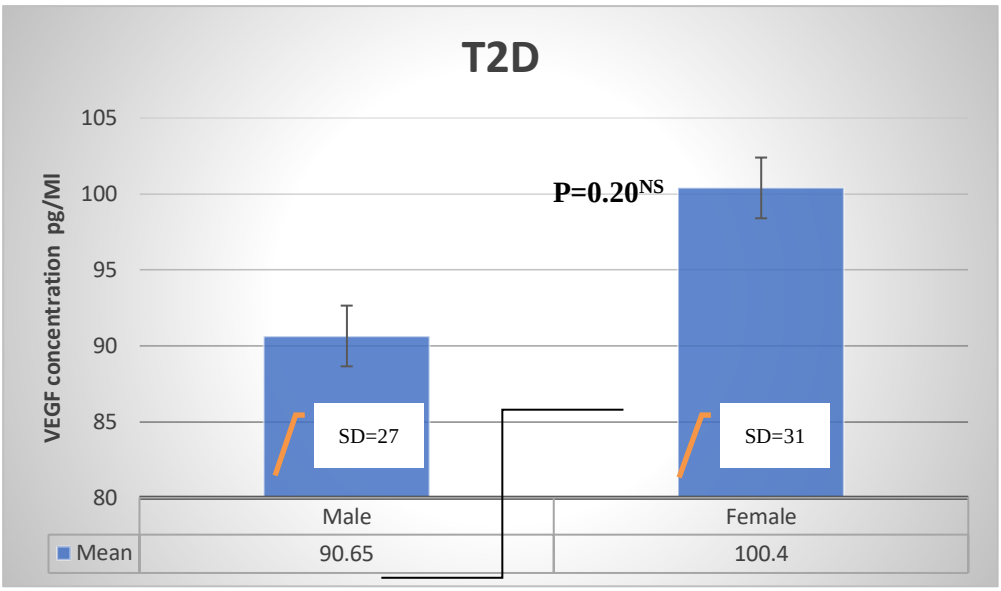

Figure (4-13): Impact of Gender on VEGF-A concentration in T2D patients

4.1.6 Distribution of Diabetic Patients According to Smoking Habit

The distribution of the diabetic patients according to smoking habit is summarized in Figure (4-14) The study included 80 patients, of whom 33 were non-smokers (15 T2D and 18 retinopathy) and 47 smokers (25 T2D and 22 retinopathy).

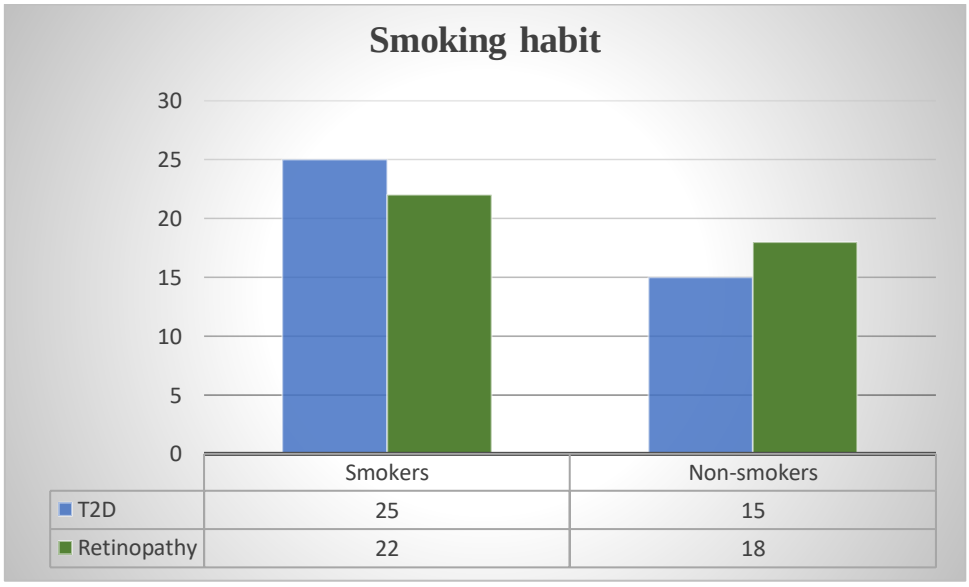

Figure (4-14): Distribution of diabetic patients according to smoking habit

4.1.6.1 Impact of Smoking habit on the glyceamic control and oxidative stress parameters in Both diabetic retinopathy and T2D patients

According to smoking habits, Table (4-10),(4-11),Figures (4-15) and (4-16). Comparison the glyceamic control and oxidative stress index parameters of non-smoker and smokers for both retinopathy and T2D patients. The table(4-10) indicates that there is a significant ($P<0.05$)increase in HbA1C and IR in smokers compared to non-smokers and a significant ($P<0.05$)increase in IS in non-smoker for patients with retinopathy. The table also showed that there was a significant ($P<0.05$)increase in FBG and IR in smoker patients compared with non-smoker in T2D. As for the oxidative stress markers, the table showed(4-11) a significant ($P<0.05$)increase in ROS was found for smokers compared to non-smokers in patients with retinopathy. A significant ($P<0.05$) increase in TAC was found in non-smokers compared to smokers, while the values of ROS and OSI in smokers increased significantly($P<0.05$) in relation to T2D.

Figure (4-15) shows a significant ($P<0.05$)increase in VEGF-A concentration in smokers compared to non-smokers in patients with retinopathy disease. while smoking had no significant ($P>0.05$)effect on VEGF-A concentration in T2D patients, as shown in Figure (4-16).

Table (4-10): Impact of smoking habit on glyceamic control parameters in retinopathy and T2D patients

Glyceamic control Parameters						
Group	**Smoking**	**FBG (mg/dl)**	**HbA1c%**	**Insulin (µIU/ml)**	**IR**	**IS**
Retinopathy	**Smoker**	261.38±78.47	10.36±2.12	28.47±10.34	15.72±8.8	0.26±0.02
	Non- Smoker	189.178±54.56	8.55±1.56	20.57±5.25	10.08±5.97	0.28±0.01
P values		0.21^{NS}	0.04*	0.29^{NS}	0.02^{*}	0.04*
T2D	**Smoker**	247.76±52.83	10.26±2.26	22.08±9.64	14.07±6.57	0.27±0.03
	Non- Smoker	193.87±62.34	9.07±2.21	18.75±8.88	10.14±4.79	0.29±0.02
P values		0.01^{*}	0.36^{NS}	0.13^{NS}	0.03^{*}	0.06^{NS}

Different small Letter refer to significant among groups comparison, similar Letters refer to non-significant differences.

SD: Standard Deviation.

NS: Non-Significant. *($P \leq 0.05$), NS ($P > 0.05$)

Table (4-11): Impact of smoking habit on Oxidative stress parameters in retinopathy and T2D patients

Oxidative Stress Parameters				
Group	**Smoking**	**TAC (mmol/l)**	**ROS (µmol/l)**	**OSI %**
Retinopathy	**Smoker**	917.63±	41.67±15.71	4.61±2.9
	Non-Smoker	1111.34±	29.65±12.97	2.81±1.62
P values		0.08^{NS}	0.021*	0.15^{NS}
T2D	**Smoker**	945.03±208.87	32.68±12.42	4.91±2.02
	Non-Smoker	1101.90±253.25	21.34±9.38	2.64±1.59
P values		0.03*	0.01*	0.011*

Different small Letter refer to significant among groups comparison, similar Letters refer to non-significant differences, SD: Standard Deviation, NS: Non-Significant. *(P≤0.05), NS (P>0.05)

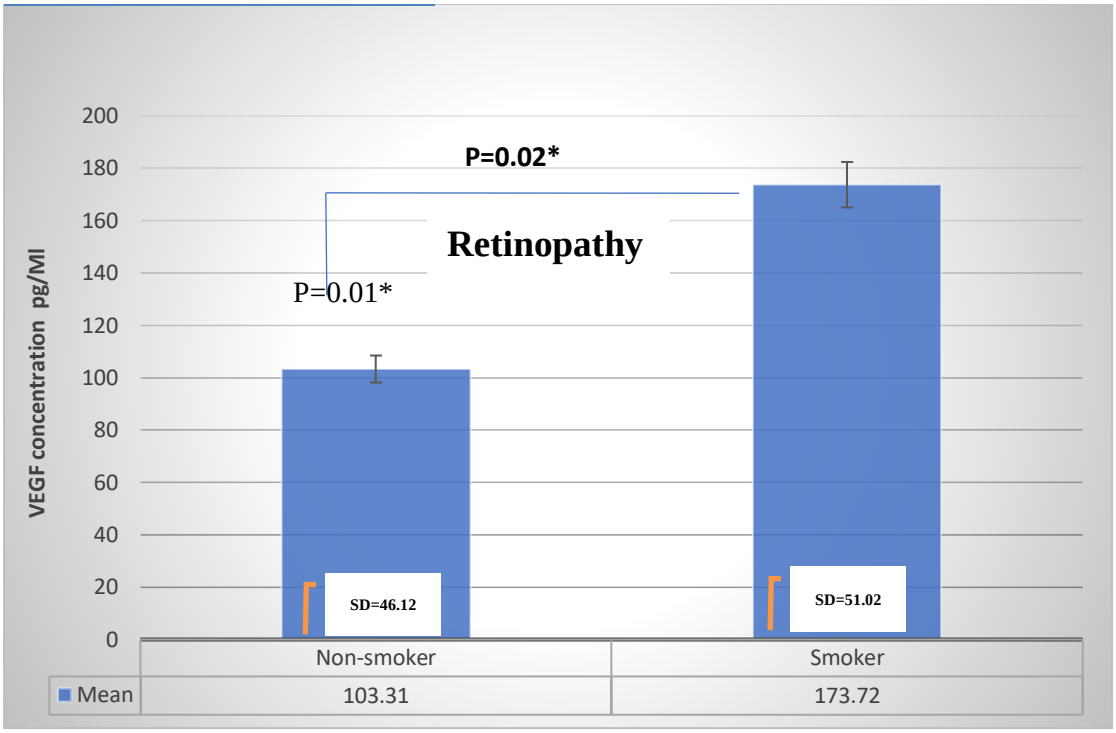

Figure (4-15): Impact of smoking on VEGF-A concentration in Retinopathy patients

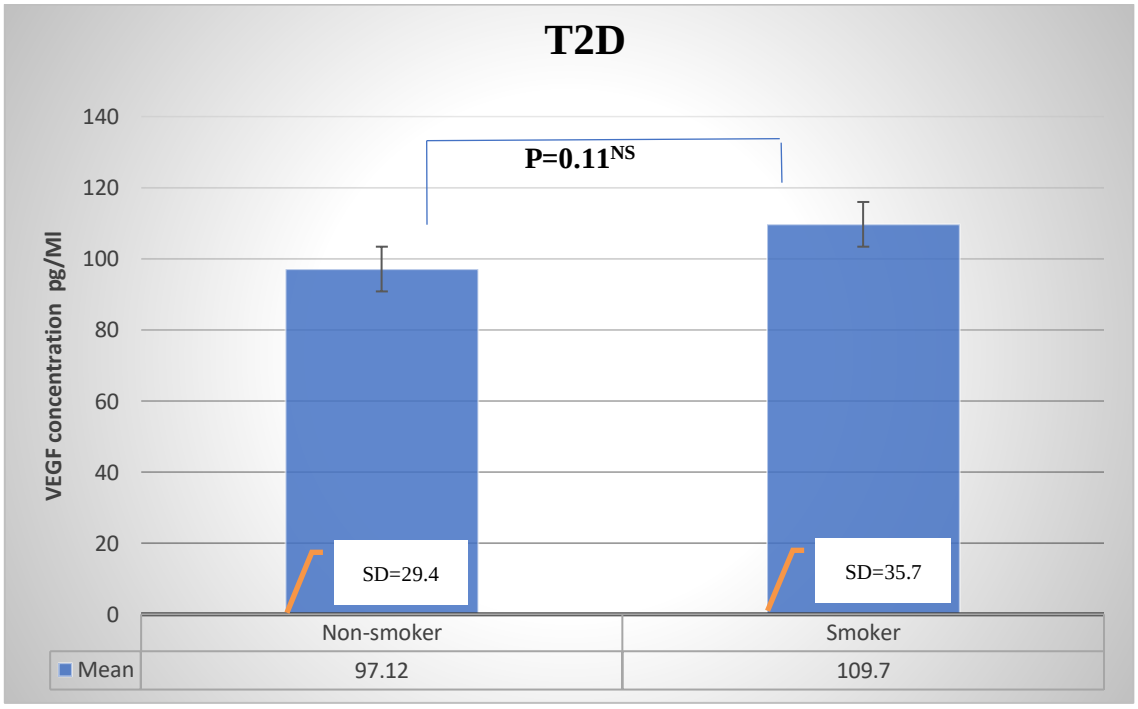

Figure (4-16): Impact of smoking on VEGF-A concentration in T2D patients

4.2 Epigenetic Study

4.2.1 DNA Extraction1

Figure(4-17) shows the pattern of DNA electrophoresis that extracted from blood of study population (Retinopathy, T2D patients and control). The DNA was once efficaciously extracted from compolete blood of samples the usage of Promega extraction kit to be used for Methylation analysis. The concentrations varied between 85 ng/µl and 200 ng/µl, yielding about 75µg DNA, (the average purity of samples used to be 1.8 for the 260/280 OD and 2.0 for the 260/230 OD).

Figure (4-17): The electrophoresis pattern of DNA extracted from blood for Diabetic patients and control, 1% Agarose ,75V, 20 mAm for 20 min. (10µl in each well). Lane 1-9 DNA from patient, lane 10-18 DNA from control, stained with red stain

4.2.2 Analysis of Global DNA Methylation

The percent of measuring global DNA methylation by estimation the 5mC % in whole genomic DNA using Methyl Flash™ Methylated DNA Quantification Kit. The analysis of Global DNA methylation revealed that Diabetic patients (both T2D and Retinopathy) have a significant ($P<0.05$) increases in mean levels of 5mC% than healthy control subjects as shown in Figure(4-18). The mean± SD of the evaluated 5mC% for patients was0.890 ±0.18 VS 0.33 ± 0.25 for health control subjects, (P= 0.002**, t=Test).

The statistical analysis showed that there was a significant ($P<0.05$) increase in the levels of methylation in Retinopathy compared with T2D patients as shown

in Figure (4-19). The mean± SD of the evaluated 5mC% for Retinopathy patients was0.96 ±0.22 VS 0.79 ± 0.13 for T2D patients (P= 0.02*, t=Test).

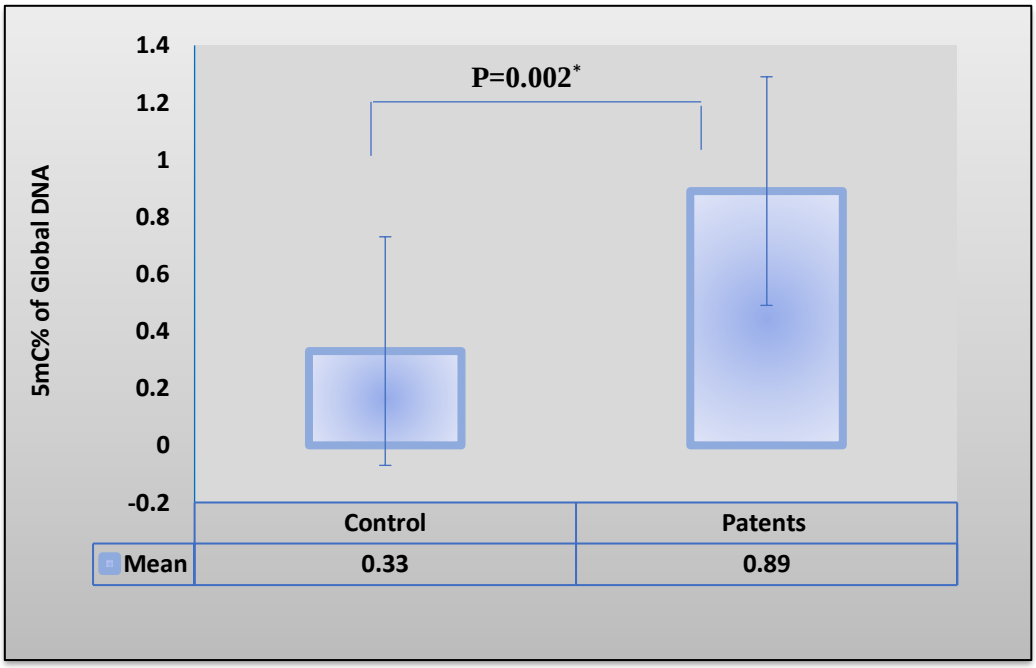

Figure (4-18): Global DNA methylation levels in Diabetic Patients and healthy controls subject

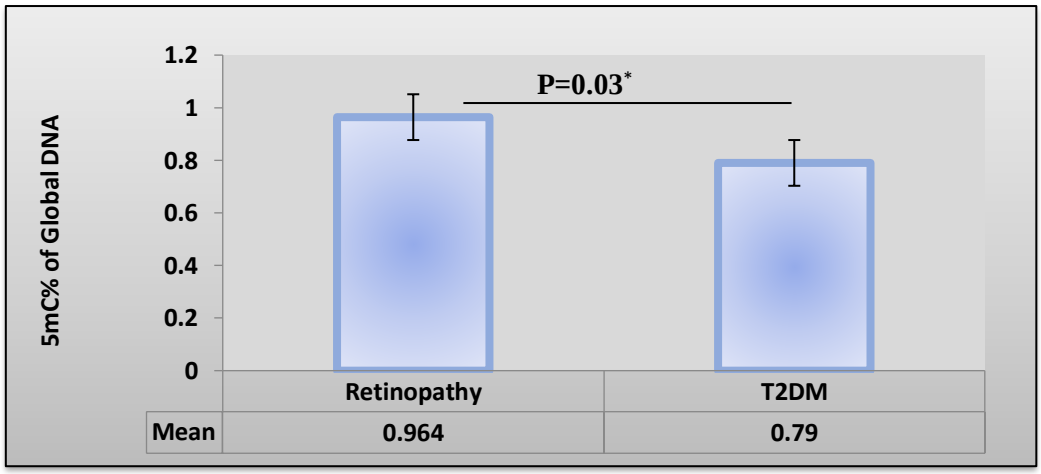

Figure (4-19): Global DNA methylation levels in T2D and Retinopathy patients

4.2.3 Differential of 5mC% of Global DNA in Study Groups According to Gender

The differences between Global DNA methylation levels according to gender in all studied groups have been outlined in Figure (4-20), that illustrates a

significant (P<0.05) increase in mean levels of 5mC% in females control 0.44 ± 0.12 compared with male0.15±0.05, P= 0.000. Also a significant (P<0.05) increase in mean levels of 5mC% in total female patients 0.99± 0.18 compared with male 0.79± 0.2, P= 004. Significant differences were also found within the retinopathy group: the levels of methylation were increased in female 1.11±0.10 than in male 0.87±0.24, P=0.02; while there were no significant (P>0.05) differences between males and females within T2D group.

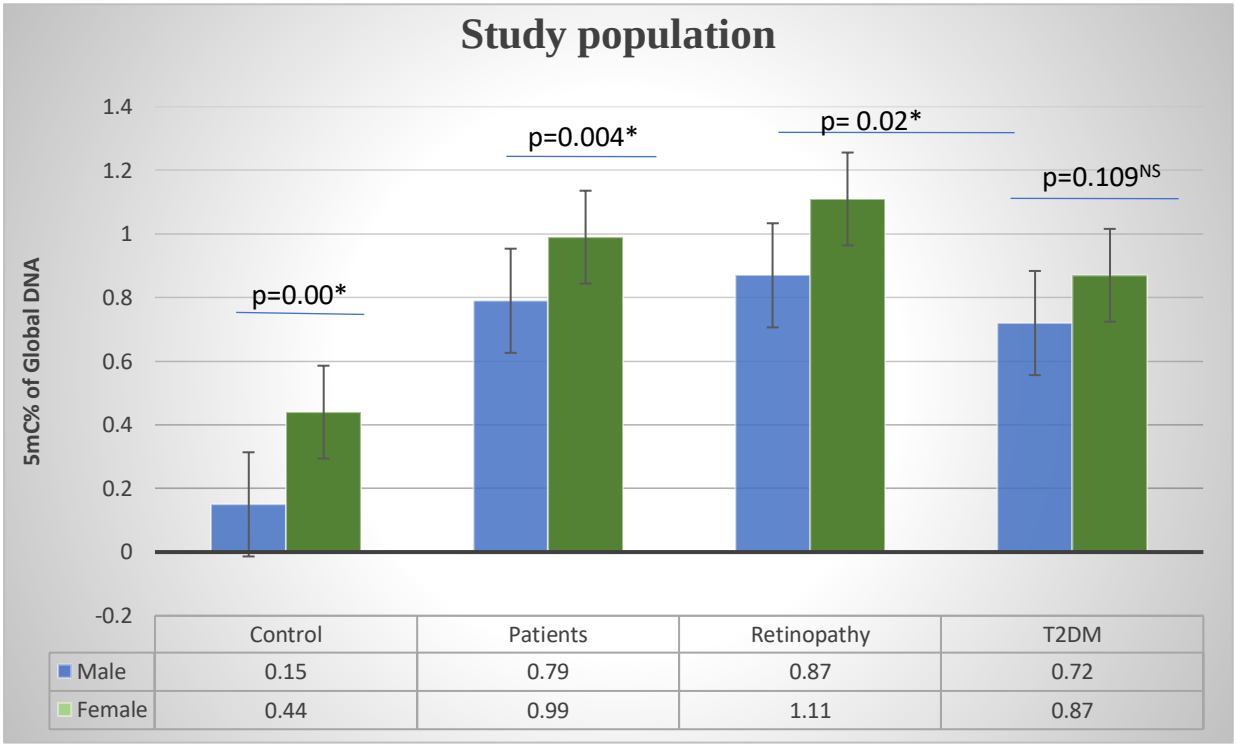

Figure (4-20): Differential of 5mC% of Global DNA in Study Groups According to Gender

4.2.4 Differential of 5mC% of Global DNA in Study Groups According to Smoking Habit

The differences between Global DNA methylation levels according to smoking in all studied groups have been outlined in Figure (4-21), that illustrates a significant (P<0.05) increase in mean levels of 5mC% in smokers control 0.52± 0.14 compared with Non-smokers0.22 ±0.13, P= 0.03*. Also a significant (P<0.05)

increase in mean levels of 5mC% in total smokers patients 1.01± 0.24compared with Non-smokers0.80± 0.14, P= 0.04*. Significant differences(P<0.05) were also found within the retinopathy group: the levels of methylation were increased in smokers0.99±0.13 than in Non-smokers0.826±0.24, P=0.04*; also a significant (P<0.05)differences were found within the T2DM group: the levels of methylation were increased in smokers 0.96±0.18 compared with Non-smokers 0.78±0.1, P= 0.00**; t=Test).

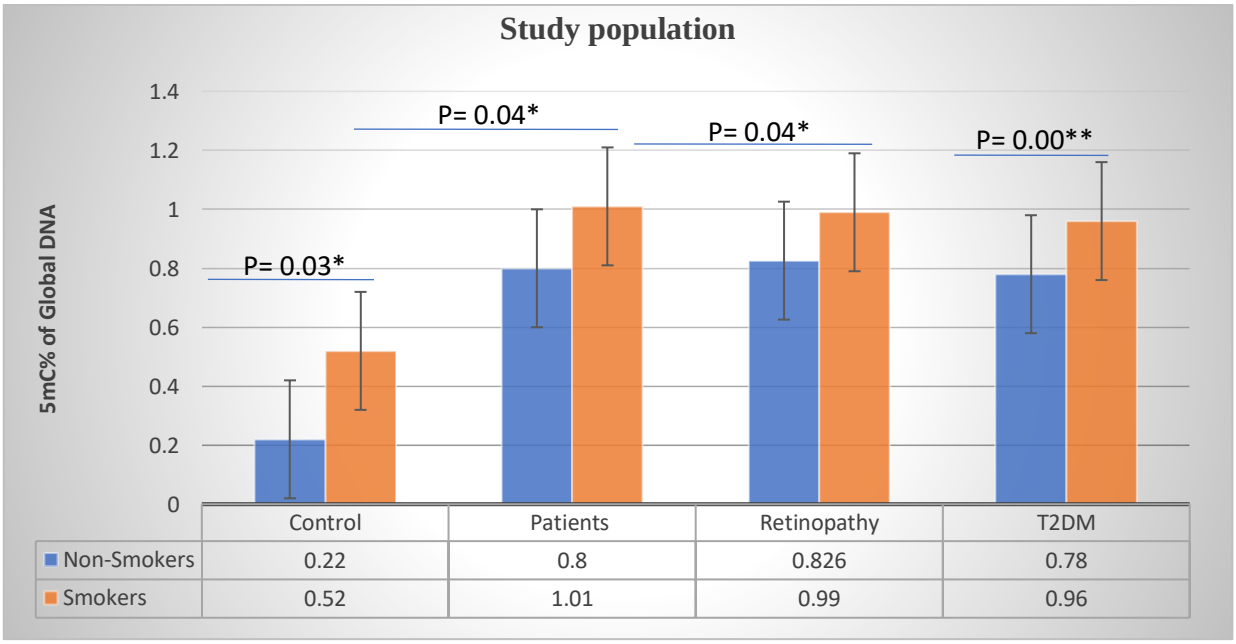

Figure (4-21): Differential of 5mC% of Global DNA in Study Groups According to Smoking Habit

4.2.5 Correlation Analysis

4.2.5.1 Correlation Between Age and Global DNA Methylation Levels in Study Groups

The correlation between age of participants subject and the levels of Global DNA methylation has been shown in Figure (4-22). The correlation and regression analysis revealed a significant (P<0.05) positive correlation between age and the percent of 5mC in all studied groups.

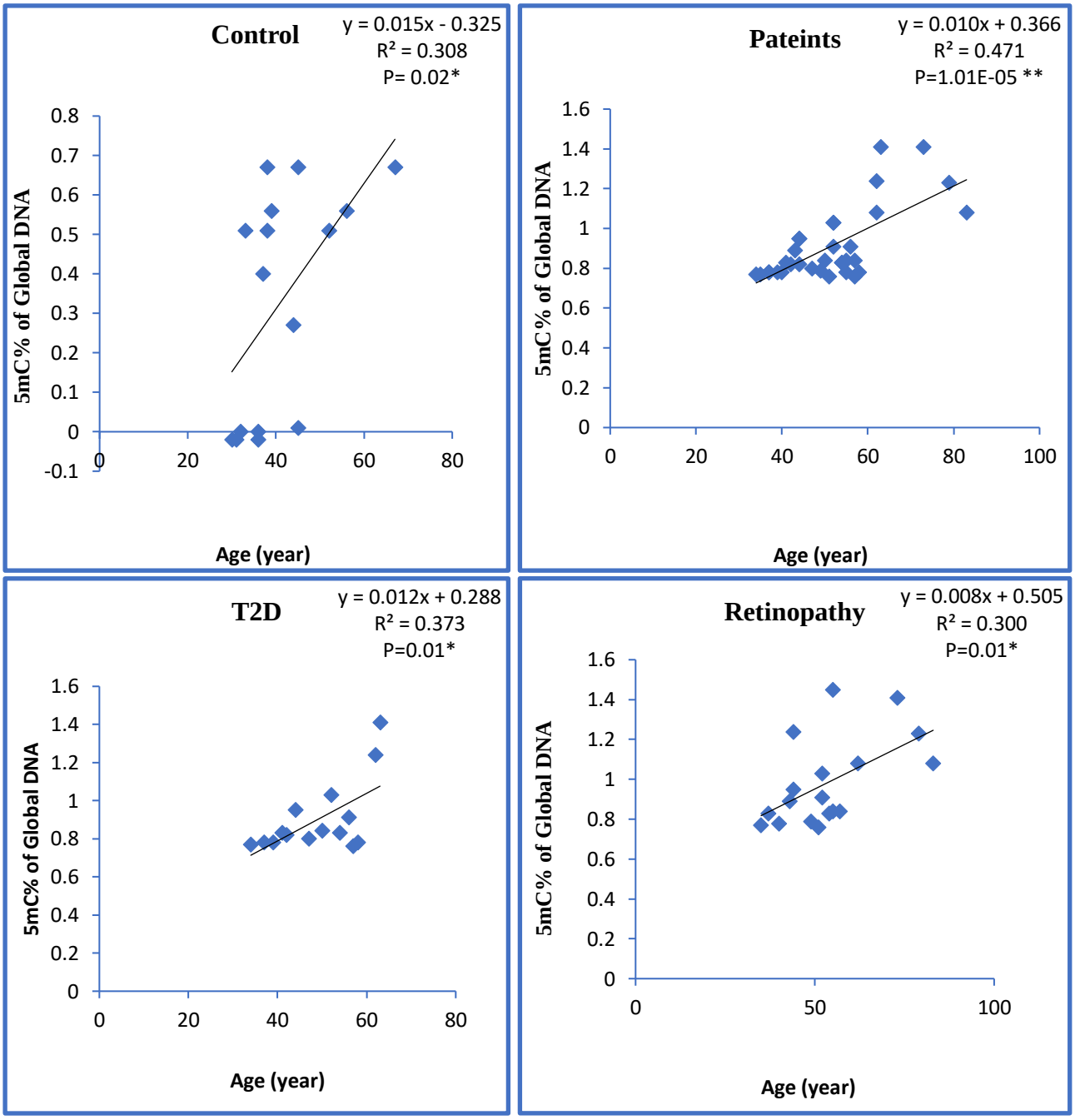

Figure (4-22): Correlation between Age and 5mC% of Global DNA in Study Groups

4.2.5.2 Correlation Between BMI and Global DNA Methylation Level in Study Groups

The correlation between BMI of participants subject and the levels of Global DNA methylation has been shown in Figure (4-23). The correlation and regression

analysis revealed a significant (P<0.05) positive correlation between BMI and the percent of 5mC in all studied groups.

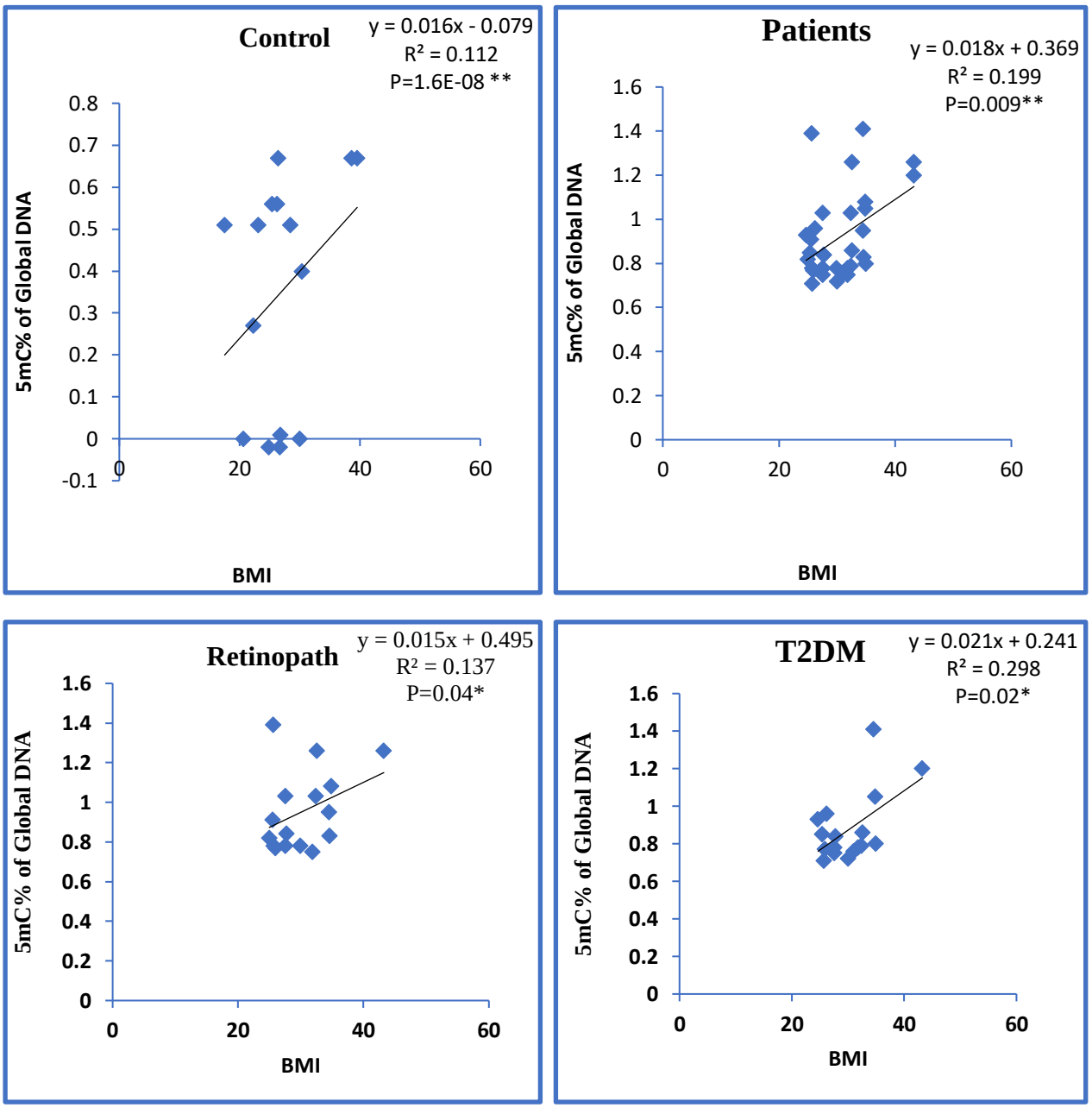

Figure (4-23): Correlation between BMI and global 5mC% in Study Groups

4.2.5.3 Correlation Between Glyceamic Control, oxidative stress Parameters and Global DNA Methylation Levels in Study Groups

Table (4-12) , (4-13) and Figure (4-24) show the correlation between the Glyceamic Control, oxidative stress Parameters and DNA methylation in total

diabetic patients and control, while Table (4-14), (4-15) and Figure (4-25) show the correlation analysis between glyceamic control, oxidative stress parameters and DNA methylation in T2D without complication and retinopathy patients.

As (4-12) Table indicates that there is a significant (P<0.05) positive correlation between DNA methylation and HbA1C, while the other parameters show non-significant (P>0.05) correlation with methylation in the control subjects. In patients, DNA methylation had a significant (p<0.05) positive correlation with FBG, HbA1C and IR in addition to has significant (p<0.05) negative correlation with IS. With regard to oxidative stress parameters, while table(4-13) the statistical analysis showed that there was no significant (P>0.05) correlation with the control, while a significant (P<0.05) positive correlation was found between DNA methylation, ROS and OSI. As for VEGF-A, Figure (4-24) shows a significant (P<0.05) positive correlation was found between DNA methylation and VEGF-A concentration in both control and patients.

Table (4-12): Correlation analysis between levels of 5mC% of Global DNA and **Glyceamic control Parameters of Diabetic Patients and control subjects**

Parameters	**5mC% of Global DNA**			
	Healthy Control		**Patients**	
	r	**P**	**r**	**P**
Glyceamic control parameters				
FBG (mg/dl)	0.13	0.17^{NS}	0.42	0.00*
HbA1C %	0.41	0.04*	0.69	0.00*
Insulin (µIU/ml)	0.03	0.23^{NS}	0.23	0.39^{NS}
IR	0.12	0.23^{NS}	0.46	0.01*
IS	-0.24	0.09^{NS}	-0.52	0.01*

r: Correlation coefficient

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

NS: non-significant

Table (4-13): Correlation analysis between levels of 5mC% of Global DNA and Oxidative stress parameters of Diabetic Patients and control subjects

Parameters	5mC% of Global DNA			
	Healthy Control		Patients	
	r	P	r	P
Oxidative stress parameters				
TAC (mmol/l)	0.11	0.31 NS	-0.08	0.53NS
ROS (µmol/l)	0.34	0.251 NS	0.59	0.023*
OSI %	0.21	0.461 NS	0.62	0.00*

r: Correlation coefficient

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

NS: non-significant

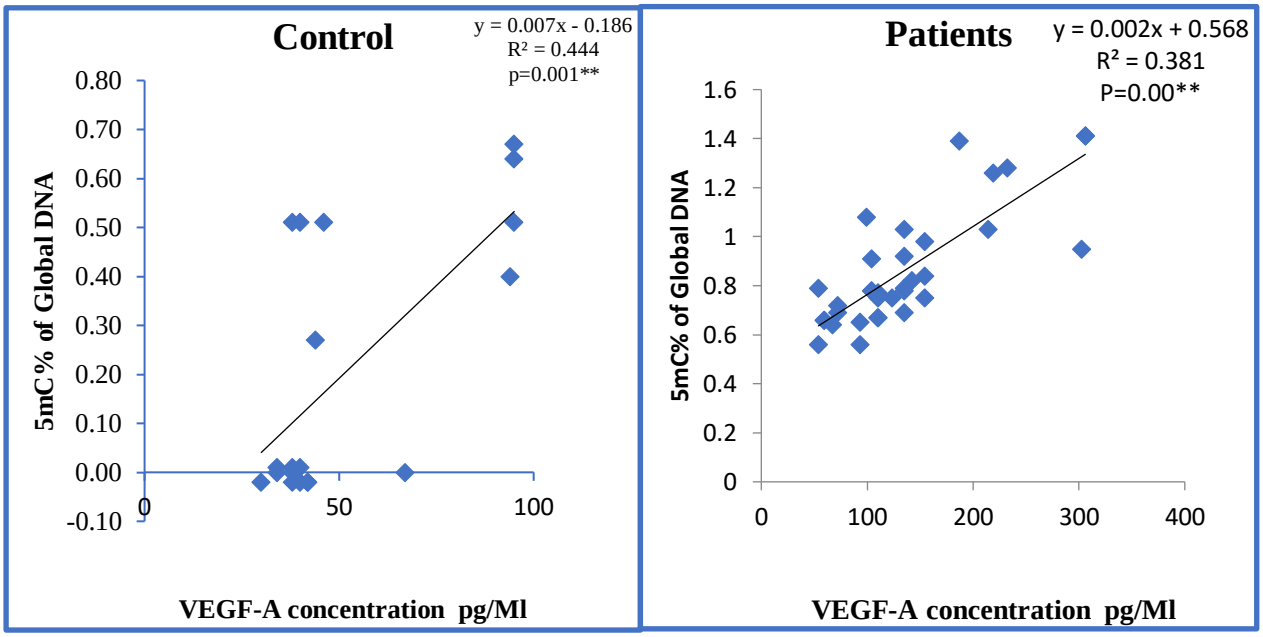

Figure (4-24): Correlation analysis between levels of global 5mC% and VEGF-A in Diabetic Patients and Control Subjects

In term of the results of the correlation analysis between glycemic control, oxidative stress parameters and DNA methylation in T2D without complication and retinopathy patients, Table (4-14) shows that DNA methylation had a significant (P<0.05) positive correlation with FBG, HbA1C and IR, in addition to has significant (p<0.05) negative correlation with IS in both retinopathy and T2D. With regard to oxidative stress parameters, while Table (4-15)the results of the statistical analysis showed that there was a significant (P<0.05) positive correlation found between DNA methylation, ROS and OSI in both retinopathy and T2D.As for VEGF-A, Figure (4-25) shows a significant (P<0.05) positive correlation was found between DNA methylation and VEGF-A in both retinopathy and T2D.

Table (4-14): Correlation analysis between levels of 5mC% of Global DNA and Glyceamic Control Parameters of T2D and Retinopathy

Parameters	5mC% of Global DNA			
	T2D		Retinopathy	
	r	P	r	P
Glyceamic control parameters				
FBG (mg/dl)	0.64	0.00*	0.53	0.00*
HbA1C %	0.55	0.00*	0.46	0.00*
Insulin (µIU/ml)	0.24	0.18^{NS}	0.19	0.39^{NS}
IR	0.49	0.01*	0.38	0.01*
IS	-0.51	0.00*	-0.44	0.01*

r: Correlation coefficient

*: Correlations significant at the 0.05 level (2-tailed)

NS: non-significant

Table(4-15): Correlation analysis between levels of 5mC% of Global DNA and Oxidative stress Parameters of T2D and Retinopathy

Parameters	5mC% of Global DNA			
	T2D		Retinopathy	
	r	P	r	P
Oxidative stress parameters				
TAC (mmol/l)	-0.06	0.61^{NS}	-0.08	0.48^{NS}
ROS (µmol/l)	0.32	0.04*	0.52	0.00*
OSI %	0.36	0.03*	0.41	0.00*

r: Correlation coefficient

*: Correlations significant at the 0.05 level (2-tailed)

NS: non-significant

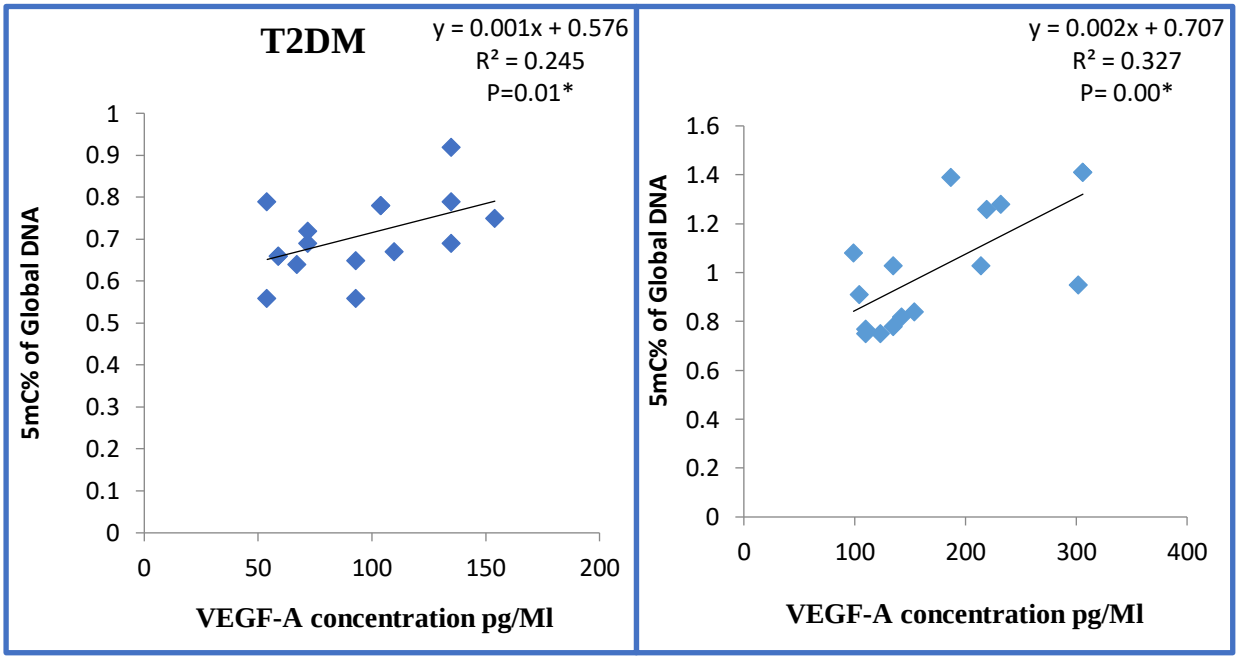

Figure (4-25): Correlation analysis between levels of global 5mC% and VEGF-A in T2D and Retinopathy patients

Chapter Five

Discussion

5. Discussion

5.1 Physiological study

5.1.1 The Descriptive data of study groups

The important characteristics for both diabetics and healthy control subjects that were taken in current study include: age, BMI, gender and smoking status. As shown in Table (4-1), there are a significant increase ($P \leq 0.05$) in the mean of age and BMI for T2D patients compared with healthy control subjects.

The aging process of the human body leads to an impairment of energy balance and abnormalities in carbohydrate metabolism. It is believed that the most essential causes of hyperglycemia are reduced insulin secretion that develops with age and increased IR (Barbieri *et al*., 2003). Members of the same species vary in the rate of aging, which correlates with their susceptibility to disease, impairment, and death (Levine, 2013). Diabetes mellitus is considered an important age-related diseases (Bahour *et al*., 2022). Regarding age, it was found that the results of the current study were logical and consistent with other studies that showed an increase in age is one of the most important risk elements for the development of T2D (Almoshabek *et al*., 2016; Al-Musawi, 2021). This relationship between the development of T2D with age may be because advancing age can mean an increase in the cumulative effect of sugar with age, in addition to the fact that some elderly people suffer from obesity. On the contrary, present study conflict with another studies as (Stoian *et al*., 2015; Hatef *et al*., 2018). That showed non-significant differences in age between T2D patients and healthy subjects.

Regarding the BMI, the current investigation showed a significant increase in T2D patients, and according to the odd ratio it is found that increased BMI in T2D patients could be an important risk factor for diabetic by 2.169-fold than control. This is concordance with previous studies (Tirosh *et al*., 2011; Twig *et al*.,

2020). That there is a study relationship between obesity and progression of T2D. Also, the finding of current study agrees with the study of Kharroubi *et al* (2015) and Al-Musawi (2021), which revealed a higher BMI for diabetic patients compared to healthy subjects. In addition, it has been proven that high BMI is an important risk factor for most of non-communicable disease such as DM, also its effect on retinopathy prove by earlier studies that identified BMI as predisposing factor for retinopathy (van Leiden *et al*., 2002; Tolonen *et al*., 2013). Lu *et al*., (2015) found U-shaped association between BMI and retinopathy in diabetes Chinese patients.

Although there was no significant effect of smoking in the current study, there is always an urgent need to address the importance of smoking cessation. Smoking will increase the danger of creating T2D. 30-40% for lively smokers in contrast to non-smokers, which indicates the need to emphasize smoking cessation as a basic public health strategy to combat the world diabetes epidemic (US Department of Health and Human Services, 2014). The World Health Organization acknowledges smoking as a preventable danger element for T2D supports keeping off smoking cessation as section of their lifestyle recommendations (WHO, 2018). The lack of significant differences may be due to the small sample size in the current study.

5.1.2 Assessment of glyceamic control and oxidative stress parameters in Studied Population

All physiological markers included in present study were assessed in both patients and healthy control (between group comparison) as well as comparing it between diabetic patients without complications and diabetic patients with retinopathy (within group comparison). Regarding to between group comparison, as indicated in Table (4-2) there are a highly significant increased ($P<0.05$) in

FBG, HbA1C, insulin and IR in T2D patients, whilst IS had a significant ($P<0.05$) increase in healthy control compared to T2D patients.

Fasting plasma glucose is a hallmark of T2D and the most broadly used diagnostic and screening check for diabetes. In general, hyperglycemia is the main feature of diabetic and its elevation may have associated with the elevation of glucagon level which involve in hepatic glucose production, the major factor that participate in fasting and postprandial hyperglycemia (Lefebvre, 2006). Cause behind hyperglycemia is IR, which increases with age (Krentz *et al*., 2013).

Other studies indicated that hyperglycemia may emerge from diverging degree of peripheral IR (Jahangir, 2019). This result is consistent with previous studies conducted on the same population (AL-Aaraji, 2017; Katulanda *et al*., 2019).

HbA1C concentration has a critical association with the incidence of retinopathy. The risk of retinopathy reduced in those patients with HbA1C levels around 7–7.5 %, and the normalization of HbA1C is one of the most dynamic systemic measures to prevent progression (Rohlfing *et al*., 2002). In June 2009, the International Expert Committee, which represents several major diabetes groups, recommended using HbA1C to diagnose diabetes (Ronald *et al*., 2011). The increase level of HbA1C in the T2D patients is suggested a poor glyceamic control as compared with healthy controls (Pieme *et al*., 2017). Similar findings were reported by other investigations (Mohsen, 2016; Al-Attaby, 2018).

It is normal for insulin levels to be higher in patients compared to healthy subjects as a result of hyperglycemic state, which could lead to a 50-fold increase in the biosynthesis of insulin (Goodge and Hutton, 2000). Hyperinsulinaemia was associated with IR, which promotes higher production of free radicals by NADPH-dependent mechanisms (Halliwell and Gutteridge, 2007). IR has serious role in the growing of hyperinsulinemia with the recompense of pancreases cells in turn out more insulin and this will lead to development of T2D (The global diabetes

community, 2015). The finding of hyperinsulinaemia of recorded in present study agrees with the results reported by Mamza *et al*., 2013, and Mohsen (2016).

The state of IR is described as an impairment of muscle glucose uptake and increased hepatic gluconeogenesis that leads to hyperglycemia, in both the fasting and postprandial states (Matthaei *et al*., 2000). Resistance of insulin is the predominant determinants of glucose metabolism disordered in T2D (Li *et al*., 2003). IR, target cells fail to reply to normal levels of insulin circulating, and consequently greater insulin concentrations are required for an everyday response (Moller and Kaufman, 2005).

According to within-group comparison, in the current study, patients with diabetic retinopathy were compared with T2D patients without complication for each of the studied parameters. The results showed that only FBG and HbA1C significantly increased in retinopathy compared to patients with T2D as displayed in Table (4-2).

The development of retinopathy is connected to risk factors including hyperglycemia. Glyceamic tests and thresholds for diagnosing diabetes are a long-running area of debate.

In a Japanese study, Nakagami *et al*., (2017), showed that HbA1C and FBG values can be considered as predictors of future development of diabetic retinopathy. The current study agrees with some studies (Rahman *et al*., 2020; Wang *et al*., 2013), which indicated that there are higher levels of FBG and HbA1C in patients with diabetic retinopathy compared to T2D patients without diabetic retinopathy.

In terms of oxidative stress, the current study dealt with the evaluation of some parameters of oxidative stress, where it was included TAC, ROSs, OSI. All these parameters showed a significant ($P<0.05$) increase diabetic patients compared with healthy control as displayed in table (4-3). Oxidative stress is a general term that mainly describes toxic effects to cells, tissues, or organs caused by ROS (Yaribeygi *et al*., 2020). Diabetics suffer from a defect in the antioxidant

defense mechanism, and free radicals and its problems may be accountable for both diabetes and its consequences (Baynes and Thorps, 1999). The synergistic effect of antioxidants is known to provide greater protection against ROSs aggression than any single antioxidant alone (Pieme *et al*., 2017).

Present finding showed a significant boost in TAC, this increase can be explained on the basis that the increase in the ROSs appear to be primarily related with an increase antioxidant levels with disease development, as soon as the antioxidant levels decrease, the disease complications will develop becuase it is providing more production against free radical aggression (Korkmaz *et al*., 2013; Pourvali *et al*., 2016). While the study of Kharroubi *et al* (2015) dissected that there was no clear explanation to the TAC behavior in diabetes patients, the current finding in line with previous studies that revealed increased TAC and ROSs in patients (Pourvali *et al*., 2016; Al-Aaraji, 2017). Whereas it did not agree with the result demonstrated by Rani and Mythili (2014).

The high prevalence of ROS lead to the activation of stress-signaling pathways and drains both enzymatic and non-enzymatic antioxidants, having a negative impact on the quality of life and lifespan of the patient. ROS play a role in multiple disease conditions including diabetes and its complications (Figueroa-Romero *et al*., 2008). Overproduction of ROS or a failure in intracellular defenses against ROS will result in pathogenesis of diabetes by causing oxidative damage to biomolecules like lipids and proteins (Bansal and Bilasquri, 2011).

Recently, Núñez-Sellés *et al* (2017) develop a procedure for calculating the OSI that associates with disease progression and oxidative stress status has been patented using a percent scale that compares the oxidative stress parameters in patients with those in healthy subjects of the same population.

According to oxidative stress within-group comparison, only significant ($P<0.05$) increase in levels of ROSs and OSI were shown in retinopathy patients compared with T2D without complications, Table (4-3). Boosted free radicals, thus increment oxidative stress, engages with pathogenesis of diabetes and related

complications like retinopathy by actuating four fundamental mechanisms, which involve: raised polyol pathway influx, raised evolution of advanced glycation end products, protein kinase C isoforms activation, and raised the action of hexoseamine pathway (Tushuizen *et al*., 2005). These aforementioned mechanisms exacerbate IR and thus increase the risk of developing retinopathy or any kind of complications associated with diabetes (Odum *et al*., 2012). The results of the current study agree with the study of (Giugliano *et al*., 1996), which showed that TAC levels were decreased in retinopathy patients, while the total oxidant status were increased according to the retinopathy status. Another investigation showed that increased markers of oxidative stress as a result of poor glyceamic control may be one of the causes of complications risk associated with diabetes, such as risk of cardiovascular diseases (Bozkurt *et al*., 2019). The majority of studies had emphasized the usage of antioxidant therapeutics for the administration of oxidative stress caused by T2D as well as by its related complications (Ahmad *et al*., 2017; Zainal, 2022).

In term of VEGF-A factor, Figure (4-1) displayed a significant ($P<0.05$) boost in diabetic patients as compared with control. Also, a significant ($P<0.05$) boost in VEGF-A levels in diabetic retinopathy as compared with T2D without complication, this finding reinforces the importance of the harmful stimulation of the VEGF-A as a result of poor glyceamic control, which stimulates many inflammatory pathways that are harmful to body tissues and exposes diabetics to many complications, the most important of which is retinopathy VEGF-A protein is one of the main proteins thought to promote angiogenesis (Ferrara, 2001).

One patho-physiologic explanation for increasing levels of VEGF-A is that persist hyperglycemia and subsequent formation of advanced glycation end products (AGE) have been shown to increase of VEGF gene transcription and mRNA production. Hence the accumulation of AGEs within tissue including the retina is suspected to upregulate VEGF production and therefore initiating and

fostering the neovascularization process (Tamarat *et al*., 2003). This interpretation is supported by the findings of a previous study, which revealed a significant association between increased levels of VEGF-A and hyperglycemia (Zafar *et al*., 2018).

The present findings in line with previous investigations that examined the relationship between VEGF-A and diabetes beside differentiating between proliferative and non-proliferative retinopathy, the consequences received from those research had been also consistent with the outcomes presented here, VEGF-A was higher in T2D with and without complications (Ozturk, 2009; Zakareia *et al*., 2010).

Much of the morbidity and mortality associated with T2D predominantly reflects its deleterious effects on micro-circulation and macro-circulation (Aiello and Wong, 2000).

Several mechanisms and molecules involved in the pathogenesis of diabetic complications, VEGF-A is one of these molecules that had a critical role with regard to retinal vascular disease as a result of its inducing-role in angiogenesis- and vasopermeability, hypoxia caused by microvascular occlusion in retinal tissues leads to the release of vasogenic mediators such as VEGF-A and thus to abnormal vascular pathologies (Fu *et al*., 2016). Results of current study are consistent with several studies (Witmer *et al*., 2003; Treweeke *et al*., 2017) that showed elevated VEGF-A levels in the serum of patients with complications from diabetes. A meta-comparison conducted in 2019 showed that serum VEGF-A levels correlate with the presence and severity of retinopathy, indicating that serum VEGF-A levels are a reliable indicator for the assessment and improvement of retinopathy (Zhou *et al*., 2019). Also, the results of the current study agree with the study of Wu *et al* (2020) who suggested that serum VEGF-A levels associated with retinopathy progression. Moreover, results obtained from previous studies (Hasanain and Alsihlawi, 2012; Abu-Yaghi *et al*., 2020; Ayan *et al*., 2023) were also in line with

results presented here where VEGF-A is higher in diabetic retinopathy patients as compared to T2D without complications patients.

On the other hand, the results of the present study do not agree with the study conducted by Swidzinska *et al.*, (2006), which documented that there were no significant differences in the level of VEGF-A between diabetic patients and healthy subjects, they attributed their findings to fact that good glyceamic management, together with metabolic side effects, can delay the onset of vascular disease in diabetic patients, (because the increased expression of VEGF-A is due to poor glyceamic control and associated metabolic complications). In spite of the conflicting findings from different projects (Ju *et al*., 2017; Ahuja *et al*., 2019), most studies indicate a higher serum levels of VEGF-A in T2D affected by retinopathy.

5.1.3 Distribution of Diabetic Patients According to Age

As shown in Figures (4-3) and (4-4), the two patients groups (retinopathy and T2D) were divided into four age categories: (35-44, 45-54, 55-64 and≥65 years), the highest percentage for retinopathy and T2D patients were within age categories 55-64 and ≥65 years respectively.

It is known that advancing age increases the risk of chronic diseases, (the most important of which is T2D), impairment, and death (Levine, 2013). In the United States, the estimated proportion of people 20 years of age or older who were diagnosed or not diagnosed with diabetes in 2005–2008 increased with age. In the 20-44 age group, it has been estimated indicates the prevalence of diabetes is 3.7%. While the percentage rose to 13.7%, the highest, in the 45–64 age group incidence was found to be 9% in the 65–year age group (Centers for Disease Control and Prevention, 2011).

Present findings agree with an investigation conducting by Cho *et al*. (2018), who found that patients aged 45-64 years were the most diagnosed age group with

T2D. Also, the current results are consistent with the study of Bahour *et al* (2022), where they suggested that majority T2D patients above the fifth decade of life, pointing to a link between cellular aging and diabetes. Varying factors involved with pathophysiology of glucose intolerance in the elderly, the primary elements are that advancing age limit the sensitivity to insulin and alter or inadequately compensate beta-cell function in the face of raised target cells resistance to insulin (Chang and Halter, 2003). A study by Szoke *et al*., (2008) shows that phases I and II insulin secretion naturally limited at approximately 0.7% per 12 months with age, and this decline in cell function characteristic quickens to double in subjects with impaired glucose tolerance. Increased oxidative stress in aged people as a results of unhealthier lifestyles, including smoking, physical inactivity, decrease hours of sleeping trigger the impairments the activity of beta-cells and IR by affecting pathways of insulin signaling (Ha *et al*., 2018).

5.1.3.1 Impact of Age on the glyceamic control and Oxidative stress Parameters in Both Diabetic Retinopathy and T2D Patients

Several studies have investigated glucose metabolism in adults with T2D, despite a relatively few have included patients > 65 years of old. An ameliorated understanding of the metabolic shifts associated with aging is crucial for the development of preemptive and curative interventions in this population.

According to result displayed in Table (4-4), all glyceamic control parameters showed a significant increase with increasing age, and this result gives a general impression of poor glyceamic control with age. This finding is in agreement with Mohammed (2014) and Joung *et al* (2018), they reveald a significant FBG increasing with age. The results of the current study also agreed with the study of Al-Attaby (2018) and Al-Musawi (2021), who indicated that the cumulative sugar and insulin levels increased with age. Not only hyperglycemia can cause complications such as retinopathy, glyceamic variability is also essential due to the complications derived from it in older patients so, it is important to

maintain as little glyceamic variability as possible in elderly patients (Zhang *et al*., 2021). Current findings agree with Mohsen *et al* (2016), who revealed an elevation in the levels of insulin in aging T2D patient and this elevation associated with insulin IR status levels.

Diabetes causes progressive retinal damage through poorly understood mechanisms. Although hyperglycemia is thought to drive retinopathy, excess glucose accounts for only 11% of variability in its risk (The Diabetes Control and Complications Trial Research Group, 1995), suggesting the involvement of other factors. Moreover, lowering glucose with insulin, insulin secretogogues, or insulin sensitizers confounds attempts to dissociate effects of glucose itself versus insulin signaling in diabetic complications.

The results of the current study are on the same track with the study of Bao *et al*., (2020) who indicated the association of retinopathy with IR in the elderly. IR is a state of inability of the target cell to respond to insulin despite the high physiological concentrations of insulin and consequently a weakness in the absorption of glucose, which leads to the persistence of high blood sugar (Al-Fartosy *et al*, 2017). This could be explaining the association of retinopathy with IR, which leads to continued damage to cellular tissues and blood vessels with persistent hyperglycemia.

Regarding to oxidative stress parameters, only ROS and OSI have asignificant ($P \leq 0.05$) differences within age categories in both retinopathy and T2D, both parameters showed a significant increasing with age. The increase in oxidative stress parameters with age is due to poor glyceamic control on the one hand, and to the decline of antioxidants with age on the other hand. Where antioxidants rise at the beginning of the disease as an attempt by the body to control free radicals, but with age and the continued disease progression, the mechanisms for producing antioxidants are depleted, which leads to an increase in

ROS and increased the OSI in patients. Similar findings support the result of the current study, which also indicated an increase in ROS levels in elderly T2D patients (Zhang *et a.l,* 2002; Pourvali *et al*., 2016).

Considering impact of age on levels of VEGF-A in studied population, the study reported an increase in VEGF-A levels at older ages, whether compared between or within groups. This relationship between age and VEGF-A can be attributed to the poor glyceamic control recorded by the study in this age group, which would stimulate the release of more VEGF-A. These results are in line with by reports from a study conducted on Jordanian population (Abu-Yaghi *et al*., 2020). However, a controversial results of age correlation with VEGF-A have been published; and this can be explained by the age range of the population under study and the distribution of participants to the different disease progression groups (Mahdy *et al*., 2010; Zehetner *et al*., 2013).

5.1.4 Distribution of Diabetic Patients According to BMI

Present study indicate that highest percent for T2D and retinopathy patients are within obesity group, it has an inverse relationship with retinopathy and T2D without complication. BMI is a popular indicator of obesity, which is considered a risk factor for T2D (Garvey *et al*., 2014). BMI in diabetic patient is one of the most important clinical parameter for their health and disease progression. The current findings that the majority weight status of T2D patients is obese is in agreement with previous studies (Leong *et al*., 2016; Sonmez *et al*., 2019). Up to now, the association between diabetic retinopathy and BMI is completely unclear. The current findings that the majority weight status of retinopathy patients is obese is in agreement with previous studies (Lu *et al*., 2015; Sarrafan-Chaharsoughi *et al*., 2018). In addition, Data with a cohort of 50,64 overweight and obese Saudi patient associated with an inverse risk of retinopathy next adjustment for age and gender have been reported (Al-Rubeaan *et al*., 2015). The exact mechanism of inverse relationship between BMI and retinopathy is not established yet, it may be

secondary to poor glyceamic control seen in diabetic patients. Also, it is considered as a risk factor for increasing IR that leads to T2D as a result of abdominal obesity such as: low muscle mass, thick subcutaneous adipose tissue (Eberhart *et al*., 2004). On the other hand, the results of the current study did not agree with a previous study, had reported no association or even conflicting results in which higher BMI is associated with lower prevalence of retinopathy (Man *et al*., 2016).

5.1.4.1 Impact of BMI on the glyceamic control parameters and Oxidative stress parameters in Both Diabetic retinopathy and T2D Patients

Pathogens or other danger factors are important in understanding the development or progression of retinopathy. BMI is a common indicator of obesity, which is regarded a risk factor for T2D (Garvey *et al*., 2014). The current study revealed the presence of significant differences for each of diabetic retinopathy patients and T2D patients between BMI groups in FBG levels, and also the presence of significant differences in HbA1C levels in diabetic retinopathy patients. These results are proportionate with former research findings that showed a positive association with BMI and higher HbA1C (Bae *et al*., 2016; Weng *et al*., 2017). But no significant difference was shown in T2D patients as in previous studies pooling data from 7 single-arm studies and 51 randomized trials demonstrated a consistent linear relationship between weight loss and reduced HbA1C between subjects for T2D who were overweight or obese (Gummesson *et al*., 2017). Additionally, previous research has shown that being overweight later in life increases the risk of T2D as being overweight in adolescence (Schienkiewitz *et al*., 2006).

Several epidemiological studies have examined the association of BMI or anthropometric parameters with diabetic retinopathy, however the conclusions have been contradictory. Recent studies in Asian populations have shown an inverse relationship between diabetic retinopathy risk and BMI, suggesting a

protective role for a higher BMI in the development of diabetic retinopathy. For example, epidemiological studies of eye diseases in Singapore found evidence of an inverse association of BMI with having diabetic retinopathy, and the Shanghai diabetes registry Database Study of 2,533 patients with T2D found that patients with weight gain they have less decrease. The risk of developing retinopathy is greater than in people of normal weight (Lu *et al*., 2015; Rooney *et al*., 2015).

Obese diabetics compared to non-obese diabetics, have higher insulin production but higher levels of IR (Jensen *et al*., 2002). Further, those with a higher BMI have been reported to demand less insulin therapies, indicating excellent beta-cell activity, which may support the idea that fat plays a protective role in retinopathy (Raman *et al*., 2010). The mechanism underlying the impact of total body fats on diabetic retinopathy is now not clear. A viable explanation for the association between weight problems and a decrease danger of retinopathy is the manufacturing of adipokines, especially adiponectin which is predominantly expressed in white adipose tissue (Joyal *et al*., 2016).

Age-related macular degeneration, retinopathy, and retinopathy of prematurity are examples of neovascular ocular illnesses for which adiponectin plays a critical function in metabolic modulations (Kaarniranta *et al*., 2012; Fu *et al*., 2015). In sufferers with T2D, adiponectin appears to modulate lipid metabolism, leading to a discount in cholesterol, and an expand in peripheral insulin sensitivity by way of controlling glycogen synthesis (Yokoyama *et al*., 2006).

Type 2 diabetes is highly associated with obesity, and the prevalence of obesity-related diabetes is expected to double to 300 million by 2025 (Dyson, 2010). This close relationship has also led to the connotation of “diabetes,” highlighting the fact that the majority of people with diabetes are overweight or obese (Hossain *et al*., 2007).

Research suggest that disturbancese in the oxidant/antioxidant balanc play an important role in the pathogenesis and complications of obesity. Excessive production of ROS and RNS alters cellular metabolism and signaling pathways (eg, NF-κB, NJK, p21RAS), causing oxidative damage to lipids, proteins, and nucleic acids (Murri *et al*., 2010 ;Li *et al*., 2015). Additionally, in obesity, ROS activates AMP-activated protein kinase (AMPK), which impairs insulin signaling in target organs as well as proliferation, apoptosis, and cell death (Rizzo *et al*., 2020). Several studies indicate that metabolic diseasese associated with obesity can occur not only due to the high accumulation of bioactive lipids (Grzegorczyk *et al*., 2018;Choromanska *et al*., 2019), but also due to disturbances in the balance of pro-oxidants/antioxidants (Yazici and Sezer, 2017). Indeed, an individuals who are obese, there has been an increase and a reduction in the antioxidant barrier (Zalewska *et al*., 2020).

Increased levels of TAC, FRAP, and DPPH may signify an improved capacity to scavenge free radicals and, thus, more efficient defense against oxidative stress in obese individuals. This is understandable considering that the first line of defense against excessive ROS/RNS generation is antioxidants. However, individuals with severe obesity showed more oxidative damage to proteins, lipids, and DNA. Increased quantities of oxidative modification products were discovered in skeletal muscle, adipose tissue, the liver, and salivary glands in addition to plasma (Fejfer *et al*., 2017; Schmatz *et al*., 2017).

In term of VEGF-A, the results of the current study are also reinforced by what was stated in the investigation of Jung *et al*., (2019), who indicated that there is a significant positive correlation between BMI and VEGF-A factor in retinopathy patients.

5.1.5 Distribution of Diabetic Patients According to Gender

The current study included taking equal numbers of samples from both sexes in order to avoid the effect of the number on the results, as one of the studies

indicates that the most important determinants of their study was the imbalance between the sexes.

5.1.5.1 Impact of Gender on the glyceamic control and oxidative stress parameters in Both Diabetic Retinopathy and T2D Patients

The current study showed that there are significant differences for gender, as it showed an increase in HbA1C levels for females compared to males in patients with diabetic retinopathy, while none of the other indicators showed any significant differences in patients with T2D.

The results of the current study indicate that more attention should be paid to the patient's gender when selecting HbA1C as a criterion in diabetes screening, as reported in previous studies (Inoue *et al*., 2012; Yang *et al*., 2015).

The results of the current study are inconsistent with studies of Rohitash *et al* (2014), and AL-Attabby (2018) which reported that men's FBG levels were significantly increased compared to women's. HbA1C levels are higher in women than in men in general, the level of hemoglobin in women is lower than that of men, and this may explain the higher percentage of glycated hemoglobin in women compared to men. The current study agrees with Hassan *et al*., (2016).

Gender differences in body composition may be due, at least in part, to the influence of sex hormones. Female sex appears to have a positive effect on insulin sensitivity, despite the higher incidence of obesity in women compared to men (Garaulet *et al*., 2000). The decline in IS with menopause, and subsequent improvement with estrogen replacement, suggests that estrogen may play a role in the IS observed in women (Moran *et al*., 2008).

With regard to markers of oxidative stress, the current study showed no significant differences in T2D patients, while in patients with diabetic retinopathy showed a significant effect in TAC for males compared to females. The result of the current study was not consistent with the study of Kharoubi *et al*., (2015). There was a significant effect of sex on TAC, as well as a significant increase in

ROS and OSI for females compared to males. It is well documented that chronic exposure to high levels of glucose increases ROS production and generates oxidative stress in islet cells. Several proposed mechanisms link hyperglycemia to increased ROS production. These mechanisms include increased glucose influx through the polyol pathway, formation of AGEs that promote oxidative stress, mitochondrial synthesis of the superoxide anion radical (O–2) and activation of the NF-κB signaling way causing an inflammatory reaction, and thus increased ROS production in phagocytes. (Gawlik *et al*., 2016).

Accordingly, the study of Jung *et al*., (2019), indicated a significant positive correlation between gender and VEGF factor in retinopathy patients.

5.1.6 Distribution of Diabetic Patients According to Smoking Habit

Smoking habit had been included in present investigation for its clinical importance, the study included 40 healthy persons, of whom 17 were smokers and 23 non-smokers, in addition to 80 patients, of whom 33 were non-smokers (15 T2D and 18 retinopathy) and 47 smokers (25 T2D and 22 retinopathy). In the current study, there was no bias in sampling or giving preference to sampling for donors participating in the study, but there was no significant difference in the ratio of smokers to non-smokers.

The reason may be due to the small size of the sample imposed by several things, including the time of the study, and the material cost.

5.1.6.1 Impact of Smoking Habit on the Glyceamic Control and Oxidative Stress Parameters in Both Diabetic Retinopathy and T2D Patients

Giving the importance of smoking habit in the development of many diseases, including diabetes and its complications such as retinopathy, the current study includes an evaluation of the effect of smoking on the criteria included in the current investigation. A previous study showed that age and smoking are among

the most important factors in the deterioration of eye health and the development of blindness (Clemons *et al*., 2005). As Table (4-10) indicated, there is a significant increase in HbA1C and IR in smokers compared to non-smokers and a significant increase in IS in non-smoker for patients with retinopathy, also that a significant increase in FBG and IR in smoker patients compared with non-smoker in T2D group. The results of poor glyceamic control in smokers T2D patients agree with several prior reports, they also showed a significant difference between smoker and non-smoker in concern to FBG, HbA1C and IR (Chiolero *et al*., 2008). Similar result has also been reported by (Al-Hashimi, 2015), who reported a higher incidence of poor glyceamic control as reflected by HbA1C among smokers T2D. While discordant with other, that found non-significant difference in the levels of FBG and HbA1C between smokers and non-smokers patients (Sari *et al*., 2018). Numerous substances included in cigarette smoke can cause Bruch's membrane and retinal pigment epithelium degeneration by increasing OSI and decreasing choroid al blood flow (Beatty *et al*., 2000).

In comparison to non-smokers, smoking raises the incidence of T2D among active smokers by 30–40%, according to a 2014 Surgeon General's study indicating the need to emphasize smoking cessation help tackle the worldwide diabetes pandemic as a crucial public health measure (US Department of Health and Human Services, 2014).

Smoking is a modifiable danger factor for numerous chronic diseases, such as CVD, cancer, chronic obstructive pulmonary illness, asthma and diabetes. However, the felled effects of smoking on diabetes are not mostly recognized (Ko *et al*., 2011). Whereas the outcomes of the present investigation concurred with the outcomes of (Foy *et al*., 2005).

Numerous Studies have demonstrated that smoking has negative consequences on diabetes mellitus that extend beyond just the macrovascular problems of the disease; nevertheless, the cause of smoking's link with diabetes

and the development of its microvascular complications have not yet been fully understood.

From the molecular point, IS may be impacted by smoking via various epigenetic pathways. The Northern Swedish Population Health Study examined 432 blood samples from participants, and the results showed that smokers had varied levels of methylation at 95 DNA methylation sites in 66 different chromosomal locations. Interestingly, genes associated with "binding to insulin receptors" and "negative regulation of glucose import" were enriched in the data set, indicating that abnormal DNA methylation may play a role in smoking's ability to cause diabetes through the impact of smoking (Besingi and Johansson, 2014). In line with this hypothesis, a recent study discovered that smoking is linked to altered methylation patterns in a number of previously known genes connected to diabetes (Ligthart *et al*., 2016).

Regarding the values of the redox indices, the results of the current study showed a decrease in the TAC values, with a significant increase in the ROS value where the new research's findings matched earlier findings in the study (Abu Khumrah, 2017). Which showed a significant evaluation of ROSs in smokers and passive smokers compared to non-smokers.

Regarding the impact of smoking status on VEGF-A levels in T2D and retinopathy patients. Interestingly, a similar finding where observed in a previous study conducting on Chronic obstructive pulmonary disease, where a significant increase in levels of VEGF recorded in smoker's patients compared non-smoking (Ugur *et al*., 2018).

Also, Jung *et al*., (2019), showed a trend to significant positive correlation found between smoking status and VEGF factor in diabetic retinopathy, (p=0.07).

5.2 Epigenetic Study

5.2.1 Analysis of Global DNA Methylation

In the present study, global DNA methylation analysis revealed that diabetic patients (both T2D and Retinopathy) have a significant ($P<0.05$) increases in mean levels of 5mC% than healthy control subjects. Also, a significant ($P<0.05$) increase in the levels of methylation in retinopathy compared with T2D patients had been reported as shown in figures (4-18 and 4-19).

Since traditional biomarkers are insufficient to explain the progression of diabetic retinopathy (Marques *et al*., 2020) and the genetic risk factors identified by GWASs do not explain more than 10% of T2D and its complication heritability (Ahmed *et al*., 2020) so, the interest in epigenetics research is growing. In addition to the epigenetic mechanisms reversible characteristics, which provide new therapeutic targets for retinal diseases, this trend has been boosted (Wu *et al*., 2021). The expression of several important genes for diabetes mellitus may be modulated by epigenetic mechanisms including DNA methylation and signaling pathway engaged in oxidative stress, inflammation, apoptosis, and aging (Gilbert and Liu, 2012). For this reason, the current study evaluated the percentage of total DNA methylation and its relationship to some factors of oxidative stress in the subjects participating in the study, to be another evidence of the importance of DNA methylation in the development of diabetes and its related complications. DNA methylation is a physiological process that regulates gene expression and, when it goes wrong, can result in illness (Jaenisch and Bird, 2003). Aberrant DNA methylation could be an aetiological factor implicated in the onset and development of T2D (Gillberg and Ling, 2015).

The present study showed a favorable correlation between DNA methylation and the development of retinopathy and T2D. The findings of the current research are consistent with previous studies that found that global DNA methylation was enhanced in diabetic and prediabetic patients compared to a control group

(Maghbooli *et al*., 2015; Chambers *et al*., 2015). It is also evident from the result of the current study that there is a significant increase in DNA methylation level in patients with retinopathy compared to patients without retinopathy, which may reinforce the importance of DNA methylation in the development of complications in diabetic patients. We conclude that the current study provides objective findings as a previous study by Maghbooli and colleagues suggested that differences in the global DNA methylation profile in T2D patients with or without retinopathy could be predictive of this complication (Maghbooli *et al*., 2015). Suggesting that an elevated potential risk factors for retinopathy may include DNA methylation status (Zhang *et al*., 2017).

5.2.2 Differential of 5mC% of Global DNA in Study Groups According to Gender

The distribution of Global DNA methylation levels according to gender revealed a significant increase in mean levels of 5mC% in female compared with male in all studied population, control, total female patients, and retinopathy group. DNA methylation is known to be influenced by gender (Schumacher and Petronis, 2006).

The current study agrees with a previous study that has demonstrated increased levels of gene specific methylation in female compared to male (Ma *et al*., 2013). Global DNA hypermethylation in female may be attributed to female-related poor glyceamic control (significant increased HbA1C) that reported in present investigation. Ahmed *et al*., (2020) stated that increased methylation status may be impute to several epigenators factors such as hyperglycemia, cigarettes smoking, nutrients and other environmental factors.

In contrast, current study disagrees with other investigations, which reported global hypermethylation in male compared with female (Hall *et al*., 2014; Al-musawi, 2021; Yadav *et al*., 2021). Although earlier research has discovered sex-specific differences in DNA methylation in several tissues, including saliva

and blood (Sarter *et al*., 2005;Boks *et al*., 2009; Liu *et al*., 2010), the majority of these studies have not connected epigenetic differences to altered metabolism. However, as this is a complicated topic, more research on how DNA methylation influence sex how it is reflected in metabolic phenotypes is required.

5.2.3 Differential of 5mC% of Global DNA in Study Groups According to Smoking Habit

Smoking is an important lifestyle factor (Breitling, 2013).Tobacco smoking is associated with an increased risk of developing T2D (Willi *et al*., 2007). Several biological mechanisms by which smoking may influence the development of diabetes have been proposed, including inflammation and the impact of nicotine on insulin impedance (Xie *et al*., 2009). However, the exact molecular mechanisms linking smoking increases the danger of developing diabetes are still largely unknown. Previous research has demonstrated that tobacco smoking has an important role in DNA methylation, which is the epigenetic mechanism of methyl group binding to nucleotides (Zeilinger *et al*., 2013; Steenaard *et al*., 2015). DNA methylation has numerous functions in the human genome, including regulating gene expression and maintaining genome stability (Jones, 2012). In line with this, previous studies have suggested DNA methylation as a possible pathway in the link between smoking cigarettes and a higher risk of developing diabetes (Besingi and Johansson, 2014). Hypothesize that exposition to naphthalene, a by-product of cigarete smoke, mutare DNA methylation. Besingi et al. reported that change in DNA methylation do not result from the basic chemical components of smoking but from the burned products generated during the smoking process. Several chemical constituents in cigarette smoke are known to be major drivers of DNA methylation change (Satta *et al*., 2008). Smoking causes inflammation and oxidative stress, which raises the risk of health issues (Kamceva *et al*., 2016). Also, cigarettes Smoke may alter methylation status during hypoxia, which in turn leads to HIF-1-dependent regulation of methionine adenosyltransferase 2a, an

enzyme that synthesizes S-adenosylmethionine, a key biological methyl at or critical donors for DNA methylation processes (Lee and Pausova, 2013). The results of the current study are in line with studies suggesting an association between smoking and global DNA methylation (Ting Hsiung *et al*., 2007). The current finding also agrees with another study which indicated that smoking has a causal impact on peripheral blood DNA methylation at several genomic loci (Li *et al.,* 2018). In addition, the results of the current study are consistent with the study of Al-Mousawi (2021), which indicated an increase in the percentage of methylation in the IGF1R gene in smokers compared to non-smokers.

5.2.4 Correlation Analysis

Correlation analysis explains the linear relationship between any two variables in terms of strength and direction. Therefore, linear correlation and regression provide useful data for evaluating the relationship between variables, whether they increased or decreased in the current study.

5.2.4.1 Correlation between Age and Global DNA Methylation Levels in Study Groups

Present study reported a significant ($P<0.05$) positive correlation between age and the percent of 5mC in all studied groups as displayed in Figure (4-22).

Generally, epigenetic marker and DNA methylation status are considered the gold standard to intend biological age (Belsky *et al*., 2020). Longevity, which is a characteristic of severe aging, is associated with age-related DNA methylation adjustments (Jones *et al*., 2015; Xiao *et al*., 2016). Existing research have indicated that epigenetic changes are an important element of the aging process (Jones *et al*., 2015). The term "epigenetics" describes how a gene's function may be altered without causing any changes to the genetic code. The epigenetic alterations of histone, non-coding RNA, and dynamic DNA methylation have all been thoroughly examined. It has been discovered that dynamic DNA methylation

changes are most closely related to the aging process (Richardson, 2003; Fraga and Esteller, 2007; Sen *et al*., 2016).

Age-dependent changes in DNA methylation globally include region-specific hypomethylation and hypermethylation (Xiao *et al*., 2016). Numerous studies have revealed a connection between DNA methylation, aging, and lifespan (Robertson, 2005). In the present study, the increased global methylation levels with age associated with marked disturbance in the most of studied parameters, which gives the impression that poor glyceamic control could manage the aberrant DNA methylation in the diabetic-related genes and lead to increased incidence of T2D and development of retinopathy complication in diabetic patients. Previous studies on global age-related DNA methylation have reported an age-related decrease in methylation based on the adult-to-elderly population. In addition, there are also reports of increased methylation associated with age and increased methylation in the first years of life (Numata *et al*., 2012;Bell *et al*., 2012). Furthermore, while many loci, such as intergenic CpG islands, display decreased methylation later in life, other loci, such as promoter-associated CpG islands, show increased methylation with age throughout the lifespan (Jones *et al*., 2012).

In contrast, Age-related global DNA hypomethylation has been documented in various research including both mice and humans (Tsang *et al*., 2016).

5.2.4.2 Correlation between BMI and Global DNA Methylation Level in Study Groups

The results in Figure (4-23) indicated a significant ($P<0.05$) positive correlation between BMI values and the percent of 5mC in all studied groups. DNA methylation status changes may occur secondary to obesity and may therefore influence the development of obesity-related diseases such as diabetes, dyslipidemia, hypertension, and cardiovascular disease. There are still significant

gaps in knowledge about how obesity and its effects are related to human epigenetic alterations (Bray *et al*., 2016). It is still unclear how much of the inter individual variation in body weight can be attributed to observable lifestyle and genetic factors. Increased global methylation levels with boost in BMI values may explains the significant increased IR in obese patients compared with other BMI groups that recorded in present study.

On the other hand, increased methylation levels in obese patients may be attributed to poor glyceamic control parameters, particularly persist hyperglycemia as well as increased levels of ROS in obese patients. Several studies have focus on the employ of DNA methylation information as diagnostic tool for the development of obesity related co-morbidities like T2D (Van Dijk *et al*., 2015). Present study agrees with previous studies that stated an association between obesity and gene specific methylation in T2D (Rohde *et al*., 2017; Al-Musawi, 2021).

It has been suggested that natural variation in DNA methylation levels may be a risk factor for certain diseases and play a role in the phenotypic variability of several traits (Zhang *et al*., 2017; Dogan *et al*., 2018). However, the relationship between DNA methylation and BMI-related sites is extra complicated. The study of Mendelson *et al*., (2017) suggests DNA methylation results may be useful in detecting negative health effects linked to BMI. The results showed a contradiction to our results, in which the global DNA methylation is negatively associated with BMI (Zhang *et al*., 2012).

5.2.4.3 Correlation between Glyceamic Control, Oxidative Stress Parameters and Global DNA Methylation

Methylation of DNA acts as an intermediary between external factors and the genome and is involved in important pathophysiological processes, including

embryonic development, stem cell differentiation, tumorigenesis and aging (Kowluru *et al*., 2016; Zhu *et al*., 2021).

It is important to study the correlation between DNA methylation and some physiological markers labels to T2D and retinopathy patients in both control and patients in general, as well as to compare this correlation between T2D patients without retinopathy and diabetic retinopathy patients in order to assess the importance of methylation status in the development of diabetes in general and its importance in the development of retinopathy caused by persistent poor glyceamic control. The results of the current study indicated that some glyceamic control parameters (FBG, HbA1C, IR) are significantly positively correlated with the global DNA methylation levels; while IS have a significant inverse correlation with global methylation status in all patients group on the one hand, and separately in T2D without complications and retinopathy on the other hand. In addition to that, control group show only a significant positive correlation between HbA1C and global DNA methylation levels as shown in Tables (4-12, 4-14). Interestingly, the results of the current study show an interesting pattern of increasing global DNA methylation levels in terms of increased FBG, IR, and impaired IS. This correlation may explain the important role of DNA methylation in predisposing patients to T2D; As well as its role in the development of retinopathy later in diabetes because it interferes with the gene expression of vital genes in the body. The current study agrees with the studies of Cai *et al*., (2020); Chen *et al*., (2021), who proved that high blood sugar is a starting point for abnormal DNA methylation in patients with T2D. The present study also in line with Wat *et al*., (2016) who stated that maintaining balanced sugar levels in the body can be a protective factor for T2D from developing retinopathy and vision loss.

Glyceamic control is a key preventable measure for curtailment the risk of retinopathy and vision loss (Wat *et al*., 2016). First, in the initial stages of T2D, re-establishment of good glyceamic control hinders retinal mitochondria from

being exposed to harmful effects when the activation of methylation factors (such as Dnmts and Tets) remain unchanged (Mishra and Kowluru, 2016). This indicates that good glucose control and maintenance at an early stage can prevent the deleterious impact of DNA methylation mechanism from damage the retina of diabetic patients. As well, even if patients do not have strict glyceamic control in the initial phase of T2D, long-term strict glyceamic control can still improve aberrant methylation status and eventually retard or stop the development of retinopathy. The significant correlation between global DNA methylation and studied physiological markers in T2D seems to be an important driver for development of retinopathy, this finding consistent with the study of Zhang *et al*., (2017), who stated that increased global DNA methylation status may be a potential risk factor for retinopathy and loss vision.

Present study is in line with previous studies which reported that gene specific hypermethylation at some diabetic related genes was positively correlated with HbA1C level, they suggested that prolonged hyperglycemia may induce aberrant DNA methylation (Yang *et al*., 2011; Yang *et al*.,2012, Al-Musawi, 2021). Also, current finding agrees with several previous studies, which reported that IR, IS, an important hallmark of T2D, have been correlated with aberrant global and specific DNA methylation (Zhao *et al*., 2012; Al-Musawi, 2021).

Regarding to oxidative stress parameters, results in Tables (4-13, 4-15) showed a significant positive correlation between each of ROS and OSI with global 5mC% levels in all patients group on the one hand, and separately in T2D without retinopathy and diabetic retinopathy on the other hand; While, control group showed non- significant correlation.

In fact, this significant correlation seems logical because during the pathological process of diabetic retinopathy, oxidative stress affects the status of methylation. On one hand, oxidative stress guided the DNA methylation. ROS are

the active intermediates of DNA methylation and can engaged in epigenetic processes by nucleophilic substitution reactions (Afanas'ev, 2014). Dnmts (the enzymes that modulate methylation status) are sensitive to redox reactions (Ziech *et al*., 2011). The production of ROS is able to activate these enzymes, promoting DNA methylation by deprotonating cytosine molecules (Afanas'ev, 2014). The function of Dnmts can be regulated by oxidative stress through a potential dual effect. The early impact prohibits the activity of Dnmts enzyme and create the highest levels of ROS, while the long term effects ameliorate the activity of Dnmts (Maugeri *et al*., 2018). On the other hand, DNA methylation boosts oxidative stress and ultimately implicates to the development of retinopathy (Kowluru and Shan, 2017). The findings of the current study agree with some previous investigations that showed a relationship between global DNA methylation and the progression of retinopathy (Maghbooli *et al*., 2015; Duraisamy *et al*., 2019). Several studies demonstrated the involvement of ROS in over gene-specific methylation under both physiologic and pathologic conditions (Afanas'ev, 2015; Sabrina *et al*., 2015). Present finding is in line with previous studies indicated the positive correlation oxidative stress parameters and over methylation status (Shrishrimal *et al*., 2019; Al-Musawi, 2021).

According to correlation with VEGF, a significant ($P<0.05$) positive correlation was found between DNA methylation and VEGF levels in both control and patients. Also, a significant ($P<0.05$) positive correlations were found between DNA methylation and VEGF factors in both retinopathy and T2D patients as shown in Figures (4-24, 4-25). VEGF is known to increase vascular leakage and angiogenesis in later stages of diabetic retinopathy, which contribute to vascular dysfunction and loss vision (Ferrara and Davis-Smyth, 1997). To our knowledge, this study is the first to show a significant correlation between global DNA methylation and VEGF in diabetic patients with and without retinopathy as well as in control subjects.

In fact, positive correlation between global 5mC % and VEGF-A factor can be explained on the grounds that increased DNA methylation in some genes can be considered as a stimulating factor for increased VEGE expression. One of these genes is Maternally expressed gene 3 (MEG3) is a Long non-coding RNAs that is related to cellular proliferation and apoptosis (Tong *et al*., 2019). It has a blocking effect on the development of diabetic retinopathy, as its over expression can decrease the pathological expression of VEGF (Di *et al*., 2022) and suppress the endothelial-mesenchymal transition (He *et al*., 2021). However, its transcription is dramatically reduced in retinopathy (Zhang *et al*., 2018). Studies have demonstrated that Dnmt1 promotes DNA methylation of the MEG3 promoter to impede MEG3 expression (He *et al*., 2021), accelerating proliferation, migration, and neovascularization in human retinal micro vascular endothelial cells (Chen *et al*., 2021).

Interestingly, Anti-VEGF therapy is useful for the treatment of retinopathy in different stages, but these anti-VEGF drugs has significant side effects, requires repeated intraocular injections, and most important of all, only about 50% of patients respond to therapy (Singer *et al*., 2016). Therefore, controlling the aberrant DNA methylation could be an alternative future treatment to the Anti-VEGF therapy. Recently, it is found that the VEGF level is under epigenetics control in an investigation in diabetic rodents (Thomas *et al*., 2017; Biswas *et al*., 2021).

Conclusions and Recommendations

Conclusions and Recommendations

Conclusions

1. Diabetic patients with and without retinopathy were associated with significantly increased levels of global DNA methylation
2. The observations of current study indicate that cigarette smoking may be involved in development of retinopathy in diabetes mellitus via the deleterious trigger of DNA methylation.
3. Global DNA methylation shows a significant positive correlation with poor glyceamic control, oxidative stress index, and the inflammatory VEGF-A factor

Recommendations

1. Highlighting another biochemical parameter such as lipid profile that associated with risk of T2D and retinopathy and evaluate their association with methylation status.
2. Examine whether DNA methylation and genetic variation interact in T2D and retinopathy patients.
3. Orientation to the study of gene-specific methylation for a key gene that contribute in the incidence of T2D and its related complications
4. Continuous encouragement to maintain an ideal body weight away from obesity in order to avoid the damage associated with obesity.
5. Shedding light on the genes and proteins that regulate the methylation mechanism in the body and their relationship to the disorder of glyceamic control in the diabetic patients.

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الخلاصة

في هذه الدراسة، قصدنا معرفة ما إذا كانت مثيلة الحمض النووي الكلية هي عامل مؤثر في اعتلال الشبكية السكري. اذ صممت الدراسة لتقييم مستويات 5mC% الكلية ودراسة الارتباط بين هذه المستويات وبعض المعلمات الفسيولوجية في مرضى السكري من النوع (الثاني) واعتلال الشبكية السكري مقارنة بالمجموعة الضابطة.

تضمنت الدراسة جمع 120 متبرعاً، منهم 40 مريضاً بداء السكري من النوع الثاني و40 مريضاً باعتلال الشبكية السكري و منهم 40 شخصًا سليمًا كمجموعة سيطرة. تضمنت الدراسة الحالية الدراسة الفسيولوجية والدراسة اللاجينية. وتضمنت الدراسة الفسيولوجية بعض الخصائص الهامة لكل من مرضى السكري والسيطرة الصحية مثل العمر ومؤشر كتلة الجسم والجنس وحالة التدخين. كما يتضمن التقييم الفسيولوجي لبعض العوامل المرتبطة بمرض السكري: نسبة الجلوكوز في الدم الصائم (FBG)، والهيموجلوبين السكري (HbA1C)، والأنسولين، ومقاومة الأنسولين (IR)، وحساسية الأنسولين (IS). فضلا عن تقييم بعض عوامل الإجهاد التأكسدي، والتي شملت: القدرة الإجمالية لمضادات الأكسدة (TAC)، وأنواع الأكسجين التفاعلية (ROS)، ومؤشر الإجهاد التأكسدي (OSI)؛ وتقييم العامل الالتهابي: عامل نمو بطانة الأوعية الدموية البشرية VEGF-A). تضمنت الدراسة اللاجينية تقييم مثيلة الحمض النووي العالمية في الدراسة.

أظهرت الدراسة زيادة معنوية ($p\leq0.05$) في متوسط العمر ومؤشر كتلة الجسم لمرضى T2D مقارنة بالأشخاص الأصحاء. بالنسبة للجنس، تم أخذ أعداد متطابقة من الذكور و الإناث.

أظهر التحليل الإحصائي أن FBG، HbA1C، الأنسولين، IR، TAC، ROS، OSI، VEGF-A تزداد بشكل ملحوظ ($p<0.05$) في مرضى T2D، بينما ينخفض IS بشكل ملحوظ ($p<0.05$) مقارنة مع أفراد السيطرة. أظهرت مقارنة المؤشرات الفسيولوجية وجود زيادة معنوية ($p<0.05$) في FBG، الأنسولين، ROS، OSI، VEGF-A في مرضى اعتلال الشبكية مقارنة بمرضى T2D.

أظهر توزيع مرضى اعتلال الشبكية و T2D حسب أعمارهم أعلى النسب في (35-44، 45-54، 55-64 و≤60 سنة). أظهر التحليل الإحصائي أنه في كلا المجموعتين (اعتلال الشبكية السكري و T2D) فإن FBG لديهم زيادة كبيرة ($p<0.05$) في المجموعات العمرية 55-64 و≤65 مقارنة مع الآخرين، في حين أظهر HbA1C والأنسولين والأشعة تحت الحمراء زيادة كبيرة في المجموعة العمرية ≤60 مقارنة

بالمجموعات العمرية 55-64 و≥65. مع الآخرين. شهدت ROS وOSI زيادة كبيرة ضمن الفئات العمرية، وكانت أعلى المستويات في ≥60 لاعتلال الشبكية وفي 55-64 و≥65 لـT2D.

وفقًا لمؤشر كتلة الجسم ، تم تقسيم المرضى إلى أربع فئات: (normal، overweight ، obese, and Morbid obesity)، . أعلى نسبة في مجموعة obese. كانت أعلى مستويات FBGفيobese و Morbid obesityفي كل من اعتلال الشبكية وT2D. أعلى مستويات HbA1C في Morbid obesityمقارنة بالمجموعات الأخرى في T2D فقط. أظهر الأنسولين وIR زيادة معنوية فيobese و Morbid obesity في مجموعة اعتلال الشبكية وT2D. انخفض IS بشكل ملحوظ فيMorbid obesity مقارنة بالمجاميع الاخرى. سجلت TAC زيادة كبيرة فيnormal, overweight في مجموعة اعتلال الشبكية. وفقط في مجموعة normal للT2D كانت أعلى مستويات ROS وOSI فيobeseو Morbid obesity في كلا المجموعتين. زاد VEGF-A بشكل معنوي في مجموعات obese وMorbid obesity مقارنة بالمجموعات الأخرى في كلا مجموعات المرضى.

أظهر تأثير الجنس على المؤشرات الفسيولوجية المدروسة ارتفاعاً معنوياً (P<0.05) في مستويات HbA1C لدى الإناث المصابات باعتلال الشبكية فقط. ارتفع TAC بشكل معنوي عند الذكور، بينما أظهر ROS وOSI زيادة معنوية (p<0.05) عند الإناث في T2D فقط. أظهر VEGF زيادة ملحوظة في الإناث مقارنة بالذكور فقط في اعتلال الشبكية.

وفقا لعادة التدخين، أظهر HbA1C، IR، ROS، وVEGF زيادة كبيرة (P <0.05) في المرضى المدخنين مقارنة بالمرضى غير المدخنين، في حين أن IS و TAC زادا بشكل ملحوظ في غير المدخنين لاعتلال الشبكية. بالنسبة لـ T2D، تم تسجيل زيادة معنوية (p<0.05) في FBG، IR، ROS، وOSI لدى المرضى المدخنين مقارنة مع غير المدخنين، في حين تم تسجيل زيادة معنوية (p<0.05) في TAC لدى غير المدخنين.

أما بالنسبة للدراسة فوق الجينيه، فقد كشف تحليل مثيلة الحمض النووي العالمي أن المرضى الذين يعانون من مرض السكري (كل من مرضى السكري من النوع الثاني والذين يعانون من اعتلال الشبكية) لديهم زيادة كبيرة (p<0.05) في متوسط مستويات 5mC% مقارنة بالأشخاص الأصحاء. كما تم تسجيل زيادة معنوية (p<0.05) في اعتلال الشبكية السكري مقارنة مع مرضى السكري من النوع الثاني.

حسب الجنس أظهرت الدراسة زيادة معنوية ($p<0.05$) في مستويات مثيلة الحمض النووي 5mC% في السيطرة على الإناث. في المرضى الإناث مقارنة بالذكور، وجد أيضًا ضمن مجموعة اعتلال الشبكية: زيادة مستويات المثيلة ($p<0.05$) في الإناث مقارنة بالذكور.

بالنسبة للتدخين، أظهرت جميع المجموعات زيادة معنوية ($p<0.05$) في متوسط مستويات 5mC%لدى المرضى المدخنين مقارنة بالمرضى غير المدخنين.

أظهر تحليل الارتباط وجود ارتباط معنوي ($p<0.05$) في مستويات 5mC% في جميع المجاميع المدروسة.

فيما يتعلق بالارتباط بين العلامات الفسيولوجية ومثيلة الحمض النووي، وجدت الدراسة وجود علاقة إيجابية كبيرة بين مثيلة الحمض النووي ونسبة HbA1C في الأشخاص الخاضعين للمراقبة. في المرضى، كان لمثيلة الحمض النووي ارتباط إيجابي كبير ($p<0.05$) مع FBG وHbA1C وIR بالإضافة إلى ارتباط سلبي كبير مع IS.أظهر ROS وOSI ارتباطًا إيجابيًا كبيرًا مع مثيلة الحمض النووي في المرضى، ويرتبط VEGF بشكل إيجابي بـ 5mC%. في كل من السيطرة والمرضى. ترتبط مثيلة الحمض النووي بشكل كبير بـ FBG، وHbA1C، وIR، بالإضافة إلى ارتباط سلبي كبير مع IS في كل من اعتلال الشبكية وT2D. أظهر ROS وOSI وVEGF وجود علاقة إيجابية كبيرة مع 5mC% في كل من اعتلال الشبكية وT2D.

نستنتج أن زيادة مستويات مثيلة الحمض النووي غير الطبيعي في الدم يمكن استخدامها كمؤشر حيوي لتشخيص اعتلال الشبكية السكري في وقت أبكر من الطرق السريرية الحالية.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية العلوم للبنات/ قسم علوم الحياة

الارتباط بين مثيلة الحمض النووي منقوص الاوكسجين في مرضى السكري من النوع الثاني المصابين باعتلال الشبكية

رسالة مقدمة الى

مجلس كلية العلوم للبنات، جامعة بابل وهي جزء من متطلبات نيل درجة الماجستير علوم في علوم الحياة

من قبل

فرح عبد الحسن حسين

(بكالوريوس علوم ، علوم الحياة، جامعة بابل، 2020)

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