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ESTIMATION ACTIVITY OF BETA -CATENIN AND BUTYRYLCHOLINESTERASE IN SERA OF DIABETIC PATIENTS TYPE2

A thesis

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By

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﴿بِسْمِ اللهِ الرَّحْمَنِ الرَّحِيمِ﴾

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DEDICATION

To the person whose name I am proud to carry....…….to my father's soul

To the soul that taught me the meaning of loss, to the one who missed her prayers and applause with joy that I accomplished this.....my mother

To the one who accompanied me every step of the way at this stage,

To my support and strength in life..... my husband

To God's gift to me and the flowers of my life ……my children

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"***In The Name of God Most Gracious Most Merciful "***

Thanks and praise be to Allah prayers and peace be upon His Prophet Muhammad and his pure good family for completing this stage.

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Researcher Sabreen

Summary

Diabetes mellitus (DM) is a metabolic disorder resulting from a defect in insulin secretion, insulin action, or both. Insulin deficiency in turn leads to chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism., and this study aims to find an association between the activity of Butyrylcolinesterase and beta catenin with some variables in diabetes mellitus type2.

The case-control study design consists of 60 diabetic patients and 30 healthy control (they were selected from Center for Diabetes and Endocrinology in Iraq Babylon City, Marjan Teaching Hospital). The groups are matched for age. And examined for FBG (fasting blood glucose), HbA1c(Glycated hemoglobin), Malondialdehyde (MDA), glutathione peroxidase (GPx), Superoxide dismutase (SOD), lipoic acid(LA), lipoic acid synthase(LIAS), mineral (Ca, k, Na), Butyrylcolinesterase (BuChE), and beta- catenin.

The results found the largest proportion of the study sample with T2 DM have hyperglycemia associated with high BMI and there no significant association between age and sex, hyperglycemia leads to decrease GPX and SOD and increase MDA levels.

There is a significant association between hyperglycemia and BuChE and higher level of BuChE among patient group. As well as there is a significant association between hyperglycemia and lipoic acid synthetase (LIAS) and also there is not association between hyperglycemia and lipoic acid.

While there is no association between hyperglycemia and beta catenin. and a significant association between hyperglycemia and calcium in patients who had lower calcium compared to healthy group.

No significant association of serum electrolytes (Ca, K, Na) and antioxidative (GPX, SOD) for good and poor HbA1c patients. while there is significant association ($P<0.001$) between FBG and HbA1c for good and bad HbA1c. Strong positive correlation between GPX with lipoic acid synthetase and Ca ion and weak negative correlation with MDA , positive correlation between BuChE with MDA and K ion and negative correlation between BuChE and SOD while no correlation found between beta catenin and other biomarkers.

Negative correlation between MDA with SOD and positive correlation between MDA with Ca and K ions, as well as there is a strong positive correlation between SOD and Ca ion and negative between K ion with Na ion.

The highest sensitivity and specificity analyses were recorded (0.60 % of GPX and 0.793 % of lipoic acid) and (0.60% of GPX and 0.767%) respectively, while lowest sensitivity and specificity are recorded to beta catenin (0.533). As well as the optimal GPX cutoff levels as identified by the maximal Youden index are 51.22 % for diabetes and healthy control .

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List of abbreviations

Abbreviation	Details
AChE	acetylcholinesterase
AIDS	Acquired Immunodeficiancy Syndrome
ALA	Alpha-Lipoic acid
APC	Adenomatous polyposis coli
BC	before Christ
BCE	Before Common Era
BMI	Body mass index
BuChE	butyrylcholinesterase
CAT	Catalase
β-Cat	β-Catenin
CKD	chronic kidney disease
CTD	carboxyl terminus domain
CVD	cardiovascular disease
DHLA	dihydrolipoic acid
DI	diabetes insipidus
DM	Diabetes mellitus
DN	Diabetic Nephropathy
ELISA	Enzyme-Linked Immunosorbent Assay
FAS II	fatty acid synthase type II
FBS	Fasting blood glucose
FPG	Fasting plasma glucose
GDM	Gestational diabetes mellitus
GMD	Glucose metabolism disorder
Gpx	glutathione peroxidase
GR	glutathione reductase
GSK-3	Glycogen synthase kinase-3
GSK3β	Glycogen Synthase Kinase3β
GSSG	Glutathione disulfide
HbA1c	Glycated hemoglobin
HIV	Human Immunodeficiacy Virus
HK	Hyperkalaemia
HPLC	High performance liquid chromatography
IDF	International Diabetes Federation
LIAS	Lipoic acid synthetase
LPO	lipid peroxidation
MDA	Malondialdehyde
MENA	Middle East and North Africa
nm	Nanometer
NTD	amino terminus domain

OGTT	**Oral glucose tolerance test**
OGTT	**Oral glucose tolerance test**
OS	**Oxidative stress**
PDH	**pyruvate dehydrogenase**
Prxs	**peroxiredoxins**
PTH	**parathyroid hormone**
RNS	**Reactive Nitrogen Species**
ROS	**Reactive Oxygen Species**
SOD	**superoxide dismutase**
T1DM	**Type 1 diabetes mellitus**
T2DM	**Type 2 diabetes mellitus**
TBA	**Thiobarbituric Acid**
TCA	**Trichloroacetic Acid**
UV.	**Ultra violet-Visible**
WHO	**World Health Organization**

Chapter One

Introduction and Literature Review

1. Introduction

1.1. Diabetes Mellitus

1.1.1. Definition

A chronic metabolic condition called diabetes mellitus (DM) have been defined as elevated glycated hemoglobin and hyperglycemia with or without glycosuria. problem with insulin production from the pancreas, insulin action or insulin resistance (or both) can lead to glucose metabolism disorder (GMD). The heart, blood vessels, eyes, kidneys, and nerves are still particularly vulnerable to injury and failure as a result from chronic hyperglycemia. These macro and micro angioplasties could be seen in people who have just been diagnosed as a result of long-term GMD[1]. Bad eating habits and a lack of physical activity are both significant modifiable risk factors for overweight and obesity in industrialized countries .Due to the social and economic burden, the incidence of DM is rising globally and become an epidemic and endemic condition [2].

According to the International Diabetes Federation (IDF), 463 million people (aged 20 to 79 years) worldwide have diabetes in 2019. by 2030 and 2045, this number is expected to rise to 578 and 700 million, respectively, with the largest increases expected in regions where economies are transitioning from low- to middle-income status.[3].

The development of disabling and life-threatening health complications, most notably microvascular (retinopathy, nephropathy, and neuropathy) and macrovascular complications leading to a2-to4-fold increased risk of cardiovascular diseases, can result from chronic hyperglycemia, which works in synergy with the other metabolic aberrations in patients with diabetes mellitus [4].Diabetes mellitus (DM) is commonest endocrine disorder that affects more than 100 million people worldwide (6% population) [5].

1.1.2. History

Diabetes was originally mentioned by the ancients. The "Ebers papyrus," Figure (1-1). an Egyptian papyrus from around 1550 B.C. , lists the essential characteristics of a polyuric state. The term "diabetes" was originally used by a Greek physician named Aretaeus of Cappadocia in the second century. In his account of symptoms, which included extreme thirst, "the melting away of flesh and limbs into urine," and brief survival, he provided the first comprehensive clinical description of the illness [6].

The word diabetes stems from the Greek word for siphon, “diabaino”, which also means “to go or run through”, alluding to the incessant flow of urine through the body [7,8].

Diabetes is dreadful affliction , not very frequent among men , being a melting down of the flesh . and limbs into urine . The patients never stop making water and The flow is incessant ,like the opening of aqueducts. Life is short ,unpleasant and painful, thirst unquenchable . drinking excessive and disproportionate to the large quantity of urine for yet more urine is Passed one cannot stop them from drinking or making water If for a while they abstain from drinking .Their mouths become parched and their bodies dry , The viscera seem scorched up the patients are affected by nausea , restlessness and a brining thirst , and within a short time , they expire.

Fig.(1.1): Clinical description of diabetes by Aretaeus of Cappadocia(2^{nd} century AD).Adopted from PapyrusNS;The History of Diabetes [8].

1.1.3. Classification[9].

Diabetes can be classified into the following general categories:

1. Type 1 diabetes (caused by autoimmune loss of beta cells, typically resulting in total insulin insufficiency)
2. Type 2 diabetes (caused by the frequent gradual loss of sufficient -cell insulin production on the background of insulin resistance)
3. Pregnancy-related type 2 diabetes (diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation)
4. Specific types of diabetes due to other by external factors, such as exocrine pancreas diseases (such as cystic fibrosis and pancreatitis), monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of young), drug- or chemical-induced diabetes (such as glucocorticoids, in the treatment of HIV/AIDS, or after organ transplantation).

1.1.3.1. Type1 Diabetes Mellitus

T1DM is an autoimmune disease, its pathogenesis is characterized by the immune system (T cells) which produces an immune response against islet, specifically destroys islet β cells, results in the abnormality of insulin synthesis and secretion, and causes the disorder of glucose metabolism of body [10].

The early onset of severe symptoms linked to an utter lack of insulin production, a propensity for ketosis, and a need for exogenous insulin to maintain life are the hallmarks of type 1 diabetes mellitus. Represents almost 10% of all diabetes cases and is one of the most prevalent chronic illnesses affecting children[11].T1DM is an autoimmune disorder in which the immune system is triggered to kill the insulin-producing pancreatic cells. This auto-immune response's origin is uncertain. T1DM is not related to alterations in lifestyle. Both a cure and a prevention are currently not possible. The primary cause of this form of diabetes is pancreatic insufficiency of insulin production,

which results from the specific loss of beta cells, with conservation within almost normal mass of alpha (glucagon) delta (somatostatin), and PP cells (pancreatic polypeptide). Measuring blood insulin levels (in individuals who have not taken the hormone externally, either fasting or basal, as to various triggers for release) can be used to illustrate this [12].

1.1.3.2. Type 2 Diabetes Mellitus

Type 2 diabetes, previously referred to as “noninsulin-dependent diabetes” or “adult-onset diabetes,” accounts for 90–95% of all diabetes. This form encompasses individuals who have relative (rather than absolute) insulin deficiency and have peripheral insulin resistance. Atleast initially, and often throughout their lifetime, these individuals may not need insulin treatment to survive [9].

Type 2 diabetes mellitus (T2DM) is a metabolic disorder caused by hyperglycemia which is the most common form of diabetes [13].

T2DM brought about by the interaction of two primary factors: impaired insulin production by pancreatic -cells and impaired insulin sensitivity of tissues. The molecular processes involved in the production and release of insulin, as well as the insulin response in tissues, must be strictly controlled in order for it to perfectly satisfy the metabolic requirement. A metabolic imbalance that results in the pathogenesis of T2DM might thus be caused by abnormalities in any of the systems involved[14]. Diabetes mellitus is a chronic, metabolic disease characterized by elevated levels of blood glucose, which leads over time to damage to the heart, vasculature, eyes, kidneys and nerves. Patients with T2DM are mostly characterized by being obese or having a higher body fat percentage, distributed predominantly in the abdominal region. The main drivers of the T2DM epidemic are the global rise in obesity, sedentary lifestyles, high caloric diets and population aging, which have quadrupled the incidence and prevalence of T2DM [15].

Type 2 diabetes is commonly manifested by middle to late aged adults (40 years); however, its prevalence is increasing in younger populations. A diabetic patient cannot metabolize carbohydrates, proteins, or fats due to improper production of insulin or resistance to insulin. Genetically predisposed obesity in people is considered to be one of the main causes of type 2 DM (T2 DM). People living with T2 DM are more vulnerable to various forms of both short and long term complications, which often lead to their premature death [16].

1.1.3.3. Gestational Diabetes Mellitus[17].

A typical pregnancy complication is gestational diabetes mellitus, in which pregnancy spontaneous hyperglycemia develops. The International Diabetes Federation (ID) current estimates from 2017 indicate that 14% of pregnancies globally and around 18 million babies yearly are affected with GDM. Overweight and obesity, a westernized diet, nutritional deficiencies, advanced maternal age, and a history of insulin resistance or diabetes in the family are risk factors. Although gestational diabetes usually goes away after delivery, it can have long term health effects including an increased risk of type 2 diabetes (T2DM) and cardiovascular disease (CVD) in the mother and future obesity, CVD, T2DM, or GDM in the child.

1.1.4. Risk Factors of Type 2 Diabetes Mellitus

1.1.4.1. Environmental Factors

Type 2 diabetes is a global public health issue, affecting 9 in100 adults worldwide in 2015 according to the International Diabetes Federation. The increasing prevalence of type 2 diabetes along with severe complications cause an massive disease and economic burden[18]. Reviews of observational studies have revealed a large number of possible risk factors for type 2 diabetes covering health status, dietary and lifestyle factors, environmental factors, and

different biomarkers, lifetime smoking, coffee (caffeine) consumption, body fat percentage, visceral fat mass, resting heart rate, childhood and adulthood BMI, alcohol consumption and Insomnia. Therefore Prevention strategies should be constructed from multiple perspectives, such as lowering obesity and smoking rates and levels, and improving mental health, sleep quality, educational level and birth weight [19].

There are several risk factors that might raise a person's chances of getting T2DM.which classified as follows:

Modifiable risk factors: include overweight and obesity, physical inactivity, high blood pressure (hypertension), and abnormal cholesterol levels .And non-modifiable risk factors: Such as socioeconomic, demographic characteristic as well as genetics, age, mental health, and history of gestational diabetes [20].

1.1.4.2. Genetic Factors

T2DM is mutagenic, which means it may be brought on by several genes. There are 36 genes that can affect type 2 diabetes. These genes can only account for 10% of the hereditary causes of diabetes mellitus. The bulk of these genes are necessary for -cell function. The 4 four hepatocyte nuclear transcription factor, the one hepatocyte, the insulin promoter, or the genetic fault at the insulin secretion site can all be involved in the genetically connected failure of -cell function [21]. It has also been shown that T2DM has a clear genetic base. In monozygous twins, T2DM concordance is around 70 % to 20-30% in dizygous twins. In research, they showed that first grade family history is associated with double risks for future T2DM [22]. Type 2 diabetes mellitus is now thought to be a disease with a strong genetic component. As a result, 35–50% of patients have diabetic relations, compared to just 15% of those who do not have this disease [20].

1.1.5. Complications of Type 2 Diabetes Mellitus

Diabetes is characterized by hyperglycemia. Long-term hyperglycemia may cause various complications and it is associated with long-term dysfunction, damage and failure of numerous organs, predominantly kidneys, eyes, blood vessels and nerves [3].

Diabetes mellitus is a complex and chronic condition that requires lifelong management. Failure to control risk factors through preventive care may lead to a host of diabetes-related complications. The majority of patients with DM (90–95%) develop type 2 diabetes mellitus. The T2DM condition is associated with a multitude of complications such as cardiovascular disease (CVD) and diabetic retinopathy, neuropathy and nephropathy[21]. Up to 25% of T2DM patients have retinopathy when they are diagnosed. One of the biggest risk factors for cardiovascular disease is diabetes in all its manifestations. Nearly 15% of stroke patients and 30% of those receiving care in cardiovascular critical care units have diabetes, and two out of every three diabetic patients will pass away from cardiovascular problems [9].

Traditional classifications of the complications of diabetes mellitus include macro vascular consequences such as cardiovascular disease (CVD) and micro vascular issues (complications affecting the kidney, the retina and the nervous system). Observational research of 28 countries (Asia, Africa, South America, and Europe) founded complications of T2DM are quite prevalent with 50% of patients with T2DM presenting with micro vascular problems and 27% with macro vascular complications [23].

1.1.6. Diagnostic Tests for Diabetes Mellitus

Hyperglycemia has a symptom of the abnormal carbohydrate metabolism that causes diabetes. So the diagnostic standard has the measurement of plasma glucose and HbA1c. However, until the underlying molecular pathophysiology of the illness is revealed, measurement of glycemia is likely to remain a crucial diagnostic modality. This technique is indirect since hyperglycemia represents the result of the metabolic derangement, not the cause [24].Whereas the capital manifestation of T2DM is hyperglycemia, other symptoms are also clinically observed. For instance, glucose detected in urine, unusual polyphagia, polyuria and polydipsia, as well as fatigue, and weight loss [25].

According to American Diabetes Association.There are three ways to diagnose diabetes are possible, (i) FPG ≥126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h. (ii) Symptoms of hyperglycemia and a casual plasma glucose ≥200 mg/dl (11.1 mmol/l). Casual is defined as any time of day without regard to time since last meal. (iii) 2-h plasma glucose ≥200 mg/dl (11.1 mmol/l) during an OGTT.The test should be performed as described by the World Health Organization,using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water as shown in Table(1-1)[26].

Table (1-1): The diagnosis of a diabetic case

FBG ≥7.0 mmol/L,Fasting = no caloric intake for at least 8 hours
HbA1C ≥6.5% (in adults)/ Using a standardized, validated assay in the absence of factors that affect the accuracy of the A1C and not for suspected type 1 diabetes
2hPG in a 75 g OGTT ≥11.1 mmol/L
Random PG ≥11.1 mmol/L Random = any time of the day, without regard to the interval since the last meal

2hPG, 2-hour plasma glucose; AlC, glycated hemoglobin; FPG, fasting plasma glucose;OGTT, oral glucose tolerance test; PG, plasma glucose [26].

1.1.7. Epidemiology of Type 2 Diabetes Mellitus

Together with obesity crisis, diabetes mellitus has emerged as one of urgent and prevalent problems in recent years. It is now the seventh leading cause of death in the USA also the rest of the world, accounting for 5.2 million deaths globally and having a mortality rate of 82.4 per 100,000 people [27]. Among the International Diabetes Federation (IDF) regions, the Middle East and North Africa (MENA) region has the second highest rate of diabetes prevalence. In MENA Region, diabetes accounts for 373557 deaths (21 countries and territories including Iraq) and an estimate of 51.8% of deaths are due to diabetes in patients aged below 60 year; this puts the region in the highest second level among IDF regions [28].

Studies have show that in the next twenty years, the incidence of T2DM is expected to increase, Particularly for those between 45 and 64 years of age. The risk factor forT2DM is age, but T2DM has become more common among children and adolescents as a result of rising rates of childhood obesity, which is a serious epidemic problem and is considering a new public health problem of large dimensions [29].

1.2. Insulin

The major purpose of the hormone produced by pancreatic beta cells is preserve glucose homeostasis. Type 1 and type 2 diabetes, metabolic conditions marked by excessive blood glucose levels are brought on by deficiencies in cell insulin production. Insulin has been a 51 amino acid dipeptide with A chain and B chain connected by two cysteine derived disulfide links[30]. There are 21 amino acids in the A chain and 30 in the B chain. In pancreatic beta-cells the short arm of chromosome 11 codes for insulin, which is made up of 100 amino acids (pre-proinsulin), including signal peptide, the B chain a connecting (C) peptide, and the A chain Figure. (1-2), shows structure of pro insulin[31].

Half-life of insulin is brief (4 to 6 min) . Insulin enters the target cell; it connects a receptor upon on surface of cells with intrinsic tyrosine kinase activity. It facilitates tyrosine auto phosphorylation of insulin receptor by binding to the α-subunit which cause phosphorylation of insulin receptor contributes to protein activation in different ways [22].

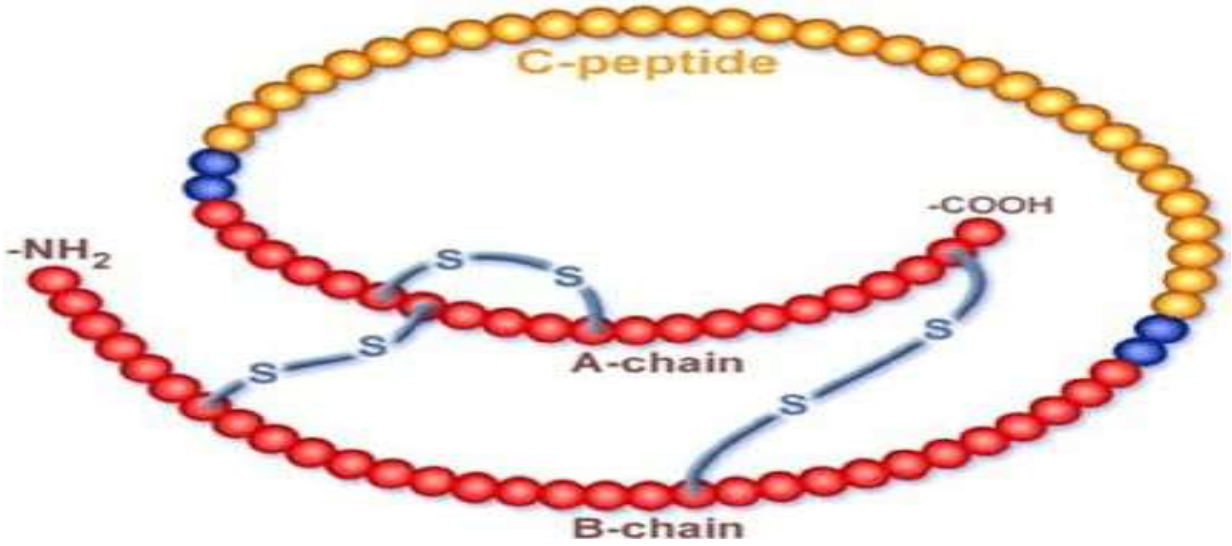


Fig. (1-2) :Structure of pro insulin showing C peptide and the A and B chains of insulin[31].

Fasting hyperglycemia which characterizes type 2 diabetes have been mostly a result of insulin's ineffective glucose-lowering activity. Therefore, it is crucial to comprehend how insulin works if we are to continue developing potent treatment approaches to treat T2D. Endocrine peptide hormone insulin interacts to receptors on target cells' plasma membranes to coordinate an integrated anabolic response to food availability [32]. Insulin continues to be the cornerstone of therapy. Newer medications complement and enhance insulin action tailored toward different mechanisms in the pathophysiology of diabetes mellitus [33].

1.3. Cholinesterase

Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are the only two members of the cholinesterase enzyme family, the form principal biological function has to control the neurotransmitter acetylcholine through hydrolysis at neuromuscular junctions, demonstrating its importance in the upkeep and functionality of neurological systems. Muscle, nerve, and hematopoietic cells produce AChE also known as "true cholinesterase," which has been regarded as one of the most effective enzymes due to its quick rate of catalysis [34]. The acetylcholinesterase (EC.3.1.1.7) (AChE), which is mainly located in the nervous system, muscles and in erythrocytes and shows particularly high affinity to acetylcholine [35]. In multicellular organisms, the neurotransmitter acetylcholine (ACh) signaling has been well recognized to have a variety of roles (control movement, heart rate, digestion, respiration, and other autonomic activities) it is facilitating communication between neurons and between neurons and muscle cells. ACh has been connected to additional processes besides these well-known ones, such vasodilation and immune cell function modification [36].

1.3.1. Butyrylcolinesterase

Butyrylcholinesterase (BuChE; EC 3.1.1.8) is a glycoprotein that has been present in most tissues with the exception of erythrocytes, including the central and peripheral nervous systems. The homotetramer is the main type of BuChE, and each monomer has 574 amino acids and nine glycosidic chains that have been attached to asparagine residues [37]. The BuChE gene has been located at 3q26.1–q26.2 on the long arm of chromosome 3. Additionally known as BuChE and other names include serum cholinesterase, plasma cholinesterase, non-specific cholinesterase, pseudo cholinesterase, and acyl choline acyl hydrolase [38]. The liver produces BuChE in its plasmatic form. Choline and aliphatic

esters, such as butyrylthiocholine, butyrylcholine, propionylthiocholine, and substances of physiological, pharmacological, and toxicological implications, can be hydrolyzed by it [37].The physiological function of BuChE is still unclear but increased activity of this enzyme has been observed in some diseases, e.g., neurodegenerative ones including Alzheimer's disease (AD) and Parkinson's disease (PD), uremia, obesity, hyperthyroidism and diabetes [38].

The overall structure of BuChE is very similar to that of ACHE .In human plasma, BuChE exists as four molecular forms. The major form of BuChE is an oligomer (340 kDa) composed of 4 identical subunits, organized as a dimer of dimers in which each dimer is composed of disulfide-bonded monomers. The natural tetramer of BuChE has not been successfully crystallized at any point in the previous 30 years[39]. It has been believed that the enzyme's high sugar content, which accounts for 25% of its mass and includes 9 extremely flexible asparagine-linked glycan chains of a complicated kind, hinders crystallization by preventing the appropriate stacking of molecules. Therefore, only a tetrameric human BuChE structure that is modeled that is identical to the tetrameric AChE structure [40]. At the bottom of a 20 deep and narrow active site canyon lies the active site of human, monomeric BuChE. Four BuChE monomers engage through a four-helix bundle in the tetramerization domain, according to a BuChE tetramer model. To create tetrameric form, a polyproline-rich peptide inserts into the four-helix bundle's middle [41]. Figure (1-3) shows the active site of butyrylcholinesterase at the bottom of a 20-Å crevice[42].

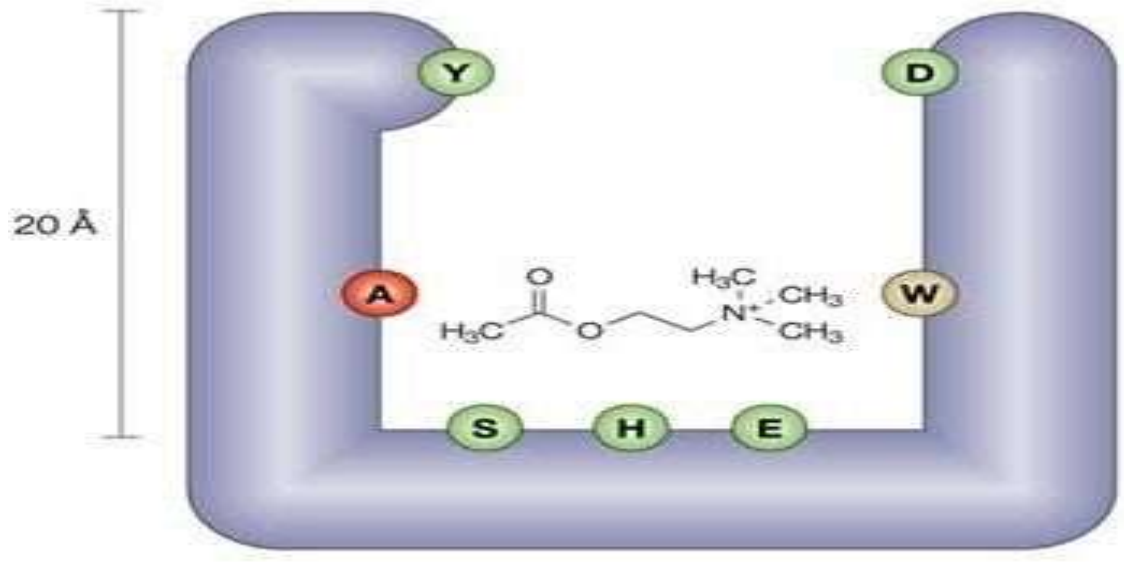


Fig (1-3): shows the active site that is the location of butyrylcholinesterase at the bottom of a 20-Å crevice. The amino acids serine (S), histidine (H), and glutamic acid make up the catalytic triad (E). The substrate's acyl group (acetylcholine is illustrated here) fits in the acyl pocket (A), while the quaternary nitrogen interacts with the anionic site produced by the amino acid tryptophan (W). Substrates are led down the active site gorge by interactions with aspartic acid (D) and tyrosine (Y)residues present at the lip of the active site crevice [42]**.**

1.3.2. Biological Properties[43-44].

Alpha glycoprotein called serum cholinesterase has been produced by the liver. mostly found in the liver as well as the central and peripheral nervous systems. It has not been repressed by high acetylcholine concentrations because of its lower affinity for the neurotransmitter. The enzyme's half-life is 12 days. BuChE typically has a value between 4,900 and 11,900 U/L. Patients with obesity, diabetes, and dyslipidemia have been found to be more active. Reduced liver damage, inflammation, and starvation are caused by the cholinesterase level . The role of BChE is still extensively discussed, it has played an important role in cholinergic mediation, it contributes to neurogenesis, and has a detoxifying effect towards different xenobiotic drugs.

1.3.3. Butyrylcolinesterase with Type 2 Diabetes

Type 2 diabetes mellitus (T2DM) is a systemic disease in which damage to multiple organs and tissues leads to microvasculature and macro vascular complications, which have a sex predominance [42]. Butyrylcholinesterase has been studied in relation to insulin resistance, cardiovascular disease, obesity, and dyslipidemia. It is a member of the esterase family of enzymes, which also includes acetylcholinesterase. There have been several theories put up on the connection between butyrylcholinesterase and type 2 diabetes. By depositing amyloid fibrils in pancreatic islet cells, butyrylcholinesterase may alter the expression of insulin resistance. Amylin was demonstrated to be affected by it [45].

In addition, serum BuChE activity correlated with serum fasting triacylglycerol concentration and insulin sensitivity in patients with type 1 and type 2 diabetes [46]. On the other hand, several reports have associated BuChE activity with obesity, coronary artery disease, adiposity, type 2 diabetes mellitus, and hepatic fat content [37].

1.4. Oxidative Stress

Oxidative stress is characterized as an imbalance between the creation and removal of reactive oxygen species (ROS) and reactive nitrogen species (RNS), favoring the synthesis of oxidants [47]. Oxidative stress is linked to the emergence of a number of metabolic, chronic diseases, or malignancies. Free radicals are unfavorable substances that are created naturally by a number of biological processes in our bodies, including respiration, digestion, alcohol and drug metabolism, and the conversion of lipids into energy [48].

The phrase "free radical" refers to a group of active intermediates (atoms or molecules) that have just one electron and have the ability to take that electron

from other atoms or substrates in order to eventually cause the substrate to oxidize. Free radicals that are overproduced *in vivo* are thought to be the start of many biological species' damages and to deactivate enzymes, which makes them the pathogenic of many illnesses [49].

The natural antioxidant mechanism in our bodies normally eliminates free radicals. Free radicals could start a chain reaction in the body that can damage cell membranes, hinder the activity of key enzymes, obstruct vital cellular processes, prevent normal cell division, degrade deoxyribonucleic acid (DNA), and prevent energy production if this system is unable to handle them [48].

In addition, long-term hyperglycemia produces free radicals to activate several pathways which might lead to diabetes [47]. Blood glucose levels are improperly controlled in both T1D and T2D, increasing to high levels over lengthy periods of time. Diabetes is characterized by this persistent hyperglycemia, which is also the primary cause of the numerous problems linked to the condition. It is generally known that oxidative stress is a major factor in the genesis and progression of diabetes, even if many elements of its physiopathology remain unknown [48].

Free radicals are transient reactive chemical substances that are byproducts of metabolism and contain one or more unpaired electrons. Free radicals come in many different forms, including ROS (reactive oxygen species), which include hydroxyl ($^{\bullet}OH$), superoxide ($O^{\bullet -2}$), hydrogen peroxide (H_2O_2), and hydrochloric acid (HOCl), as well as RNS (reactive nitrogen species), which include nitric oxide (NO), nitrogen dioxide (NO_2), and the non-radical peroxynitrite ($ONOO^-$)[50]. All of these are linked to diabetes and diabetic complications. For example, reactions to anoxia, anti-infection, and mitosis need low to moderate quantities of free radicals, but high levels of ROS and RNS cause oxidative stress, which in turn causes damage to bio

macromolecules [51]. These reactive species are not necessarily a threat to the body under normal physiological conditions , but when the body fails to remove them to a certain degree, oxidative stress stimulates the formation of atherosclerotic plaques and increases the risk of coronary artery disease, type 2 diabetes mellitus (T2DM), and atherosclerosis [52].

1.4.1. Lipid Peroxidation

High intracellular levels of reactive oxygen species (ROS), which may damage proteins or lipids and DNA this a sign of oxidative stress. Particularly, ROS leads to the oxidation of polyunsaturated fatty acids in membrane lipid bilayers, which eventually results in the creation of aldehydes, which are thought to be hazardous messengers of oxidative stress and have the ability to spread and aggravate oxidative damage. Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), two of the aldehydes generated by lipid peroxidation (LPO), have drawn the most interest since MDA is frequently employed as a marker of oxidative stress because it is produced at high levels during LPO [53]. Lipid peroxidation is the removal of electrons from lipids by free radical species such oxy, peroxy, and hydroxy radicals, which produces reactive intermediates that can be subjected to additional processes. Phospholipids are directly harmed by lipid peroxidation, which can also serve as a cell death signal and cause programmed cell death. Additionally, oxidized phospholipids typically influence pro-inflammatory alteration and can play a significant role in a variety of inflammatory diseases [54]. Lipid peroxidation is one of the most substantial reactions of free radicals.Tissue destruction by oxidative stress can be determined by the final end products of lipid peroxidation, such as malondialdehyde (MDA) [55].

Reactive oxygen species produced from the direct oxidation of glucose or weakened antioxidant defenses are directly linked to persistent hyperglycemia in

the diabetic condition. One effect of elevated ROS is lipid peroxidation, which occurs when ROS attach to unsaturated fatty acids and change their structure, producing malondialdehyde, a crucial indicator of oxidative damage [56].

1.4.2. Malondialdehyde

One of the most common and effective products for analyzing unsaturated fatty acid peroxidation processes is malondialdehyde (MDA). MDA levels rise during inflammatory processes and if the body's reduction system fails to neutralize it, can harm the structure and functionality of cell membranes. It is a biomarker of oxidative stress that is created by ROS [57].Free radicals and other oxidants hydrolyze poly unsaturated fatty acids molecules present in cell membrane leading to formation of Malondialdehyde. MDA is very reactive and is one of the electrophilic compound that can cause toxic damage in tissues. MDA induces damage to tissues by formation reactive compounds which are considered as advanced lipoxidation end products [58].

Chronic hyperglycemia is associated with oxidative stress. Oxidative stress can be determined by several ways, such as MDA. MDA is one of recommended marker for oxidative stress [59]. MDA may attach to a variety of functional molecular groups, including proteins, lipoproteins, RNA, and DNA. In vitro and in vivo, the presence of MDA in biological materials can be used as a potent indication of lipid peroxidation for a range of diseases. [21]. Basically, MDA is a colorless organic compound in a liquid state, with the chemical formula $CH_2(CHO)_2$ and is a highly reactive compound that is denoted as the enol [65].A MDA formation route is recognized in Figure(1-4)[53].

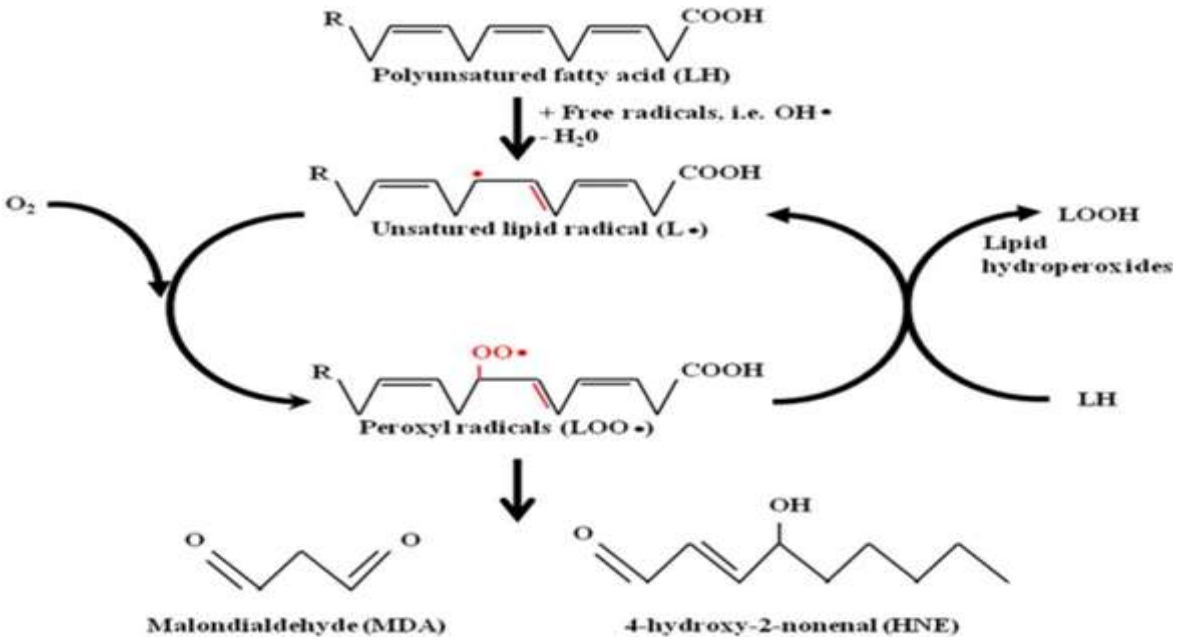


Fig.(1-4): Malondialdehyde (MDA) and 4-hydroxynonenal (HNE) formation from polyunsaturated fatty acid [53].

1.5. Antioxidant

A substance is described as an antioxidant if it directly scavenges ROS, up-regulates antioxidant defenses or prevents ROS formation. Antioxidant substances could scavenge free radicals, lengthen shelf life by delaying the lipid peroxidation process and shield the organism from the harmful effects of free radicals and ROS. Both lipid peroxidation and the progression of many chronic illnesses are slowed down by them [61]. Different types of intracellular ROS can be scavenged by antioxidant enzymes and non-enzymatic antioxidants . Antioxidants may be enzymatic or non-enzymatic. One of the enzymatic antioxidants playing an important role in the first line of defense is superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and peroxiredoxins (Prxs) These enzymes neutralize hydrogen peroxide (which is produced by SOD), yielding water (catalase, glutathione peroxidase) with oxygen molecule (by catalase) [62]. The non-enzymatic

substances taking part in the first line of defense belong to preventive antioxidants were found in blood plasma which were represented by ceruloplasmin, ferritin, transferrin and albumin. Non-enzymatic antioxidants, which are represented by molecules with the capacity to quickly inactivate radicals and oxidants, are the second line of defense against ROS [63]. Repair processes against ROS and free radical damage make up the third line of defense. Enzymatic antioxidants offer this type of defense because they can restore DNA and proteins that have been damaged, combat oxidized lipids, halt the chain reaction of peroxyl lipid radicals, and restore molecules and cell membranes that have been damaged [64]. The main characteristic of a compound or antioxidant system is the prevention or detection of a chain of oxidative propagation, by stabilizing the generated radical, thus helping to reduce oxidative damage in the human body [65].

1.5.1. Enzymatic Antioxidants

These cellular antioxidants which are present in the biological system and play a crucial role in maintaining redox balance are known to maintain stability and regulate the growth of free radicals induced by the action of oxidative stress in the body. This stability in homeostasis has been caused. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) are the key enzymes that make up this endogenous antioxidant system [66].

1.5.1.1. Superoxide Dismutase

The first enzyme in the detoxification process and most potent antioxidant in the cell has been superoxide dismutase (SOD, EC 1.15.1.1). It has a crucial endogenous antioxidant enzyme that serves as a part of the body's initial line of defense against reactive oxygen species (ROS). It facilitates the dismutation of

two superoxide anion molecules ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) and molecule oxygen (O_2), reducing the potential danger of the superoxide anion. Because SOD is a metallic enzyme, it needs a metal cofactor to function [67]. Cu/Zn SODs, Mn SOD/Fe SODs, and Ni SODs are three kinds of SOD that have developed in various species that include various catalytic metal ions. SOD enzymes have unique subcellular localizations in addition to requiring metal ion cofactors. Only Mn SODs and Cu/Zn SODs (in the cytoplasm and extracellular space) are expressed by eukaryotes (in the mitochondria) [68]. Superoxide is thought to function where it is formed since it cannot easily travel across cell membranes and has a limited lifespan. H2O2, in contrast, is a more adaptable signaling molecule since it is uncharged, more stable, and can readily move through membranes [69]. The enzyme has anti-inflammatory properties and can stop precancerous cell alterations. As a person ages, their body's natural SOD levels decline, making them more susceptible to illnesses linked to oxidative stress. An important factor in the development of diabetes and its consequences is increased oxidative stress. Diabetes often results in an increase in ROS creation and impaired antioxidant defenses because diabetes's continuous hyperglycemia encourages the synthesis of ROS from multiple sources. O_2– to H_2O_2 conversion is catalyzed by SOD [70].

$2O_2^{\cdot-} + 2H^+ + SOD \rightarrow H_2O_2 + O_2$

In turn, H_2O_2 can be removed by the other enzymatic antioxidant systems, such as CAT and GPX [48].

Endothelial cells create more $O_2^{\cdot-}$than usual when blood sugar levels are high. Glyceraldehyde-3-phosphate dehydrogenase, a crucial enzyme in the glycolytic process, can be inhibited by an excess of $O_2^{\cdot-}$[68].This results in a switch to other alternative routes of glucose metabolism, the buildup of glucose and other intermediate metabolites of this pathway, and an increase in the synthesis of advanced glycation end products [70].

1.5.1.2 Glutathione Peroxidase

The initial description of glutathione peroxidase (GPx; EC 1.11.1.9) dates back to 1957. One of the most essential elements of the antioxidant system that works with the main antioxidant molecule glutathione is glutathione peroxidase. Eight GPx isozymes have been discovered so far; GPx-1, -2, -3, -4, and -6 include selenocysteine in their catalytic site, whereas GPx-5, -7, and -8 employ cysteine instead [71]. GPx catalyzes the reduction of H_2O_2 or organic peroxide (ROOH) to water or alcohol.

$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$

$RCOOH + 2GSH \rightarrow ROH + GSSG + H_2O$

These actions took place in the presence of GSH, a tripeptide present in cells in large amounts that is transformed into GSSG by the catalytic reaction of GPx [72].

Glutathione peroxidase are ubiquitous enzymes, appearing in prokaryotic or eukaryotic microorganisms, invertebrates, vertebrates and plants [73]. The activity of GPx is dependent on selenium, which is an essential mineral in the diet due to the requirement for selenocysteine in some selenoproteins. GPx promotes protection against reactive oxygen species (ROS) and reactive nitrogen induced cell damage. Because of its antioxidant activity [74]. The enzyme's most important function is to prevent the oxidation of lipids, which protects cells from oxidative stress [67]. It plays an important role in the maintenance of the reactive oxygen species (ROS) metabolic balance in vivo. In the absence of this antioxidant enzyme, a buildup of ROS ensues that is known to damage DNA, proteins, and lipids. The most prevalent selenoperoxidase, GPx1, may be found in almost all cells. Much more GPx2 is present throughout the gastrointestinal system, particularly in the intestine. GPx3 is mostly found in the kidney compared to other organs, however it is also present as a glycoprotein in extracellular fluids [75]. Antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase (GPx) as well as endogenous metabolites

are responsible for scavenging and breaking down ROS to less or nonreactive products [76].

1.5.2. Non-Enzymatic Antioxidants

The second line of defense against ROS involves non-enzymatic antioxidants that are represented by molecules characterized by the ability to rapidly inactivate radicals and oxidants [63]. Non-enzymatic antioxidants like glutathione (GSH), vitamins(such as vit. C, vit. E, vit A),Flavonoid compounds, phenol compounds, Lipoic acid , uric acid (UA) and Allantoin (the product of uric acid under influenced of oxidative stress reaction) were extensively reported to have positive modulatory effects against oxidative stress (OS) deleterious potential by neutralizing the ROS [60]. Although there is an increase in the formation of free radicals and reactive oxygen species (ROS), living things have developed defenses against oxidative stress. These defenses include internal metabolic responses and external elements like pollution, smoking, and UV radiation (OS) The endogenous enzymatic antioxidant system is the primary mechanism, although dietary non-enzymatic antioxidants are also crucial to thwart this process. The primary dietary sources of antioxidants such vitamin C, vitamin E, and carotenoids are founds in fruits and vegetables. These substances help to reduce the risk of a number of non-communicable chronic illnesses and aging by shielding cells from oxidative damage brought on by free radicals [77].

1.5.2.1. Lipoic Acid

A naturally occurring dithiol molecule called lipoic acid (LA), or 1,2-dithiolane-3-pentanoic acid, is produced enzymatically in the mitochondria from octanoic acid and cysteine (as a sulfur donor). One of LA's antioxidant abilities is its capacity to replenish endogenous antioxidants like glutathione and vitamins E and C as well as to directly scavenge ROS. Dihydrolipoic acid (DHLA) is regarded as an antioxidant molecule even in its reduced state [78]. A

chain of eight carbon atoms, two of which are oxygen atoms in the carboxylic group and two of which are sulfur atoms in the terminal section, combine to produce this tiny amphipathic molecule Figure(1-5)[79].

Alpha lipoic acid, which is abundant in cellular membranes as well as the cytoplasm, it is a good antioxidant. Dihydrolipoic acid (DHLA) reduced form of ALA, is thought to be a more potent antioxidant than ALA and has the ability to work in concert with other antioxidants including GSH, ascorbate, and tocopherol [72]. Structure of alpha lipoic acid shows in Figure (1-5) [79].

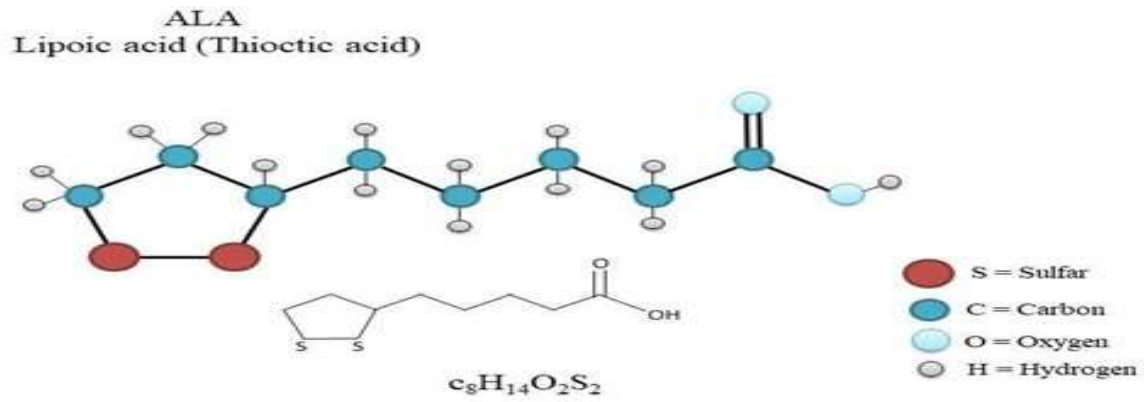


Fig. (1-5): Structure of alpha lipoic acid [79]

Reed discovered alpha lipoic acid in 1951 as an acetate substitute, and it was first used in a therapeutic setting in 1959 to treat acute death cap (also known as Amanita phalloides) poisoning [80].

The disulfide bond in the molecule contributes to the stability and redox-dependent control of these multienzyme complexes as well as serving as a source of reductive potential needed for the catalysis of mitochondrial 2-ketoacid dehydrogenases. For the oxidation of carbohydrates, amino acids, and other fuels as well as the control of mitochondrial redox balance, these capabilities make lipoic acid crucial for cell proliferation[81].

A cofactor for several enzyme complexes involved in cellular energy production, alpha lipoic acid has significant functions in a variety of chemical

processes. Additionally, it binds with proteins to create covalent bonds and may be medicinal. It produces two optical isomers, R- and S- lipoic acid, by virue of its solitary chiral center and asymmetric carbon atom S and R enantiomers, which are seen of as mirror reflections of one another, are the two enantiomer forms of ALA(Figure 1-6) [81]. The R isomeric form of ALA is naturally present, but the S isomeric form is created by chemical processes. Both S and R enantiomers are present in equal amounts. The R enantiomer is naturally formed inside of living things by making covalent connections with proteins, and foods are a natural supply of it.

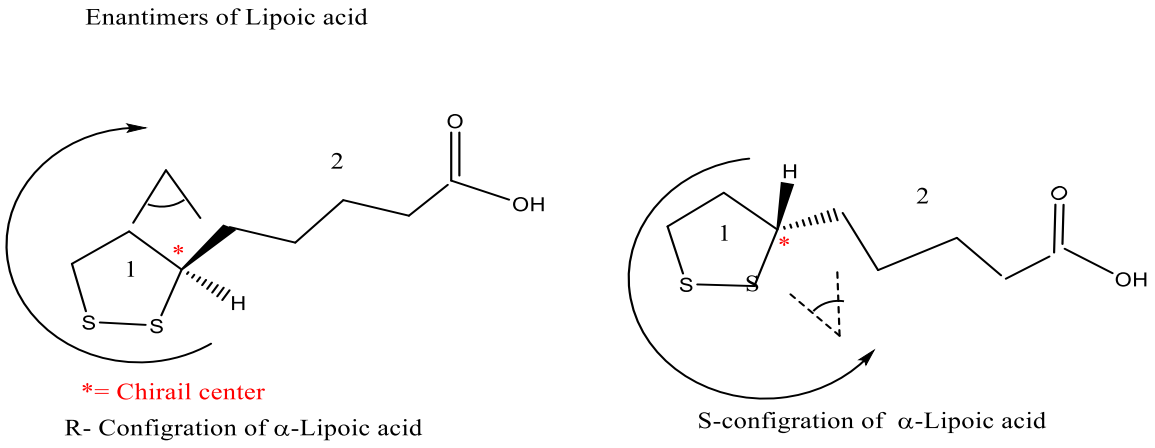


Fig. (1-6): The chemical structure of optical isomers of alpha lipoic acid ALA

Alpha lipoic acid is found in many vegetables (spinach, broccoli, tomato, Brussels sprouts, and rice bran),meats and entrails (e.g., liver and kidney) in lipoyl lysine form (ALA) with binding lysine residues [80].

A molecule called lipoic acid has two thiol groups that can be reduced or oxidized. The reduced form of LA is known as dihydrolipoic acid(DHLA), while its oxidized form is typically referred to as -lipoic acid or simply lipoic acid. The most common form of LA that interacts with ROS is DHLA, however LA in its oxidized state also has the ability to neutralize free radicals. Due to the stress of the S-S-C link in the heterocyclic disulfide circle, it is very reactive. When heated over its melting point (47.5°C) or when it is dissolved in a neutral

solution under the influence of light, LA, which is generally stable as a solid, polymerizes [82].

Its antioxidant properties can prevent and reduce micro and macro-vascular complications in diabetic patients, and that's why it considered as an effective therapy of diabetic nephropathy (DN). Experimental studies have shown that ALA will improve blood flow to the nerve, reduce oxidative stress and enhance distal nerve conductivity [78].ALA mimic insulin stimulating glucose uptake, the up regulation of adiponectin and the activation of adenosine monophosphate activated protein kinase (AMPK) in white adipose tissue, and has been shown to prevent hyperinsulinemia and insulin resistance. In general, ALA efficiently reduces the level of glucose in type 2 diabetes patients by possibly enhancing a production of the insulin from a pancreas [83].

The unstable, highly reactive chemicals known as free radicals, which are byproducts of both healthy and stressed-out cell activity, cause cellular damage that alpha-lipoic acid combat. Endogenous antioxidants including glutathione, vitamin E, and vitamin c can be recovered to it. It works well for a variety of medical disorders, including heart disease, type 2 diabetes, and liver illness [84]. LA and DHLA contribute to the synthesis of insulin. According to research by Goraca [82]. LA improves glucose absorption in both insulin-resistant and insulin-sensitive muscle tissue.

Numerous studies have demonstrated that ALA can aid in the management of diabetes, particularly type 2 diabetes mellitus (T2DM). Insulin resistance and insufficient insulin secretion are two features of T2DM, a metabolic condition. Free radical and inflammatory cytokine production can both be increased by hyperglycemia. ALA lessens insulin resistance while enhancing the efficiency of insulin and the delivery of glucose to the cells[83]. According to Gorca, ALA

increases the efficiency of insulin, facilitates the transfer of glucose into cells, and lessens insulin resistance[82].

1.5.2.2. Lipoic Acid Synthetase

Lipoic acid synthetase(LIAS, EC: 2.8.1.8),contains two [4Fe-4S]-type iron–sulfur (FeS) clusters, which are sulfur donors and essential for the enzyme activity [85]. Lipoic acid is formed in mitochondria by a series of reactions involving the mitochondrial fatty acid synthase type II (FAS II). Via FAS II, octanoic acid is synthesized in an acyl carrier protein (ACP)-bound fashion. This reaction is catalyzed by the enzyme lipoic acid synthetase, which is a highly conserved enzyme found in prokaryotes and eukaryotes [86].

Lipoic acid synthetase (LIAS) gene was identified at human chromosome 4 in the p14 band Lipoic acid synthetase (LIAS), a key enzyme for the synthesis of lipoic acid. For the action of various mitochondrial complexes, including the pyruvate dehydrogenase (PDH) and the alpha-ketoglutarate dehydrogenase (KGDH) complexes. Lipoic acid is an essential cofactor that binds and is required for the activity of several mitochondrial complexes such as the pyruvate dehydrogenase (PDH) and the alpha-ketoglutarate dehydrogenase (KGDH) complexes. PDH transforms pyruvate into acetyl-CoA as the Krebs cycle's gate key [87].The LIAS gene product is one of the mitochondrial proteins that is capable of alpha-lipoic acid synthesis. Alpha-lipoic acid shows an obvious antioxidant activity [88].

Both deficiencies in the production of the [4Fe-4S] FeS cluster cofactors and mutations in the structural gene LIAS can result in Lipoic acid synthetase deficiency.All lipoyl-containing enzymes, including the 2-oxoacid dehydrogenases and the Glycine cleavage system, have reduced lipoylation when LA synthetase is defective [85].

1.6. β-Catenin

β-Catenin is a protein containing a central armadillo domain, amino terminus domain (NTD) and carboxyl terminus domain (CTD). Historically, β-catenin was initially characterized in a seminal screening of genes required for embryonic development in Drosophila [89].

The β-catenin protein has been consisting of three domains: an N-terminal domain (~130 aa), a central domain (residue 141-664 a.a.) made of 12 Armadillo repeats and a C-terminal domain (~100 aa). The central domain of the protein is the arm repeat domain, forms a rigid rod-like structure and interacts with many binding proteins. β-Catenin protein was first discovered as a component of the adherent junction. Later, it was considered as a multitasking protein involved in transcription and cell adhesion [90].

1.6.1. β-Catenin Roles

β-Catenin is a protein that serves two distinct roles depending upon its subcellular location

1. β-catenin in the nucleus is a critical effector of the Wnt signaling pathway [91].The general role of β-catenin is to facilitate the recruitment of the mediator complex essential for RNA Polymerase II-mediated transcription and the binding of histone acetyltransferases to open chromatin, thereby promoting the recruitment of general transcription factors to target gene promoters [92].
2. β-catenin at the membrane is a component of the adherens junction (AJ) complex in association with cadherins within the AJ complex. β-catenin acts as a bridge between α-catenin and Cadherins to maintain proper cytoskeleton structure, cell–cell interaction, and cell signaling [91].

1.6.2. β-Catenin functions

Main function of beta catenin as both an adherents junction protein for cell to cell adhesion and a signaling molecule for canonical Wnt signaling, Growth, differentiation, and cell polarity depend on beta-catenin. Additionally acts as a transcriptional coactivator for the production of Wnt target genes. A massive multiprotein assembly known as the β-catenin destruction complex targets the non-junctional pool of cytoplasmic β-catenin for proteolysis in the absence of an external Wnt stimulus [91]. When it was discovered that β-catenin accumulates and target genes are improperly activated as a result of mutations of the adenomatous polyposis coli protein linked to familial and sporadic colon cancers, the significance of β-catenin destruction was first revealed [93]. Also, elevated levels of this protein, which are often brought on by mutations, are associated with the advancement of pathology in epithelial carcinomas. Obligatory phosphorylation of N-terminal residues, followed by ubiquitination and proteasome-mediated destruction, is the main mechanism controlling -catenin abundance. Cancer-associated mutations that inhibit or impede proteasome-mediated degradation, leading to increased carcinogenesis, underscore the significance of proteasome-mediated degradation in regulating -catenin activity [94].

β-Catenin is recruited into a destruction complex that contains APC and AXIN, which facilitates the phosphorylation of β-catenin by casein kinase 1 (CK1) and then GSK3β. This leads to the ubiquitylation and proteasomal degradation of β-catenin [95].

1.6.3. Wnt/ β-Catenin Singaling Pathway

The first Wnt protein, a group of secreted signaling glycoproteins, was identified in Drosophila and named Wingless. Wingless signaling induces nuclear translocation of an intracellular protein named Armadillo or β-catenin in vertebrates. The Wnt pathway is involved in development, tissue specification, and cellular migration [96].

By controlling cell fate, differentiation, apoptosis, polarity, and migration, the Wnt/-catenin pathway controls a number of physiological processes, including embryogenic development, adult tissue homeostasis, wound healing, and stem cell maintenance[97].

The Wnt family of proteins belongs to a group of secreted lipid-modified glycoproteins, which contain highly conserved cysteine residues in its amino acid sequence. There are 19 different Wnt proteins that have been identified in humans and mice. The best characterized Wnt signaling pathway is the canonical Wnt/β-catenin signaling pathway [98]. β-catenin is the key mediator of the canonical Wnt pathway. In the absence of a Wnt ligand, β-catenin is degraded by a destruction complex [99].

Wnt signaling is a growth control mechanism that may influence a wide range of biological functions, from adult homeostasis through development and evolution canonical (β-catenin-dependent activity) and non-canonical (β-catenin-independent activity). Wnt pathways are the two branches of Wnt signaling. Twelve incomplete Armadillo repeats make up the core region of β-catenin, which is surrounded by several domains in the N- and C-terminial, respectively [100].

Function of the canonical Wnt pathway mainly controls cell proliferation, whereas the non-canonical Wnt pathways regulate cell polarity and migration, and the two main pathways form a network of mutual regulation. Wnt signalling

plays an important role in the self-renewal of some tissues in mammals. For example, the Wnt signaling pathway is related to the development and renewal of small-intestinal epithelial tissue and promotes the differentiation of Paneth cells at the base of the crypt. In addition, the Wnt signaling pathway is closely related to liver metabolism and regeneration, lung tissue repair and metabolism, hair follicle renewal, hematopoietic system development, and osteoblast maturation and activity [101].

The extracellular Wnt ligands are typically highly conserved, and they bind to membrane receptors in an autocrine/paracrine manner to activate the canonical Wnt pathway. Once engaged, the usual Wnt pathway enhances -catenin's stability and moves it to the nucleus, which eventually promotes the activation of genes involved in cell growth, survival, differentiation, and migration [102].

The extracellular signal, membrane segment, cytoplasmic segment, and nuclear segment are the four segments that make up the Wnt/-catenin pathway. Wnt proteins, including as Wnt3a, Wnt1, and Wnt5a, play a major role in the transmission of extracellular signals[98]. The Wnt receptors LRP5/6 and Frizzled (a unique sevenfold transmembrane receptor Frizzled protein) are mostly found in the cell membrane segment. β-Catenin, DVL, glycogen synthase kinase-3 (GSK-3), AXIN, APC, and casein kinase I make up the majority of the cytoplasmic section (CK1). The majority of the β-catenin in the nuclear section translocate to the nucleus [102].

Inhibiting the axin degrade some destruction complex, a multiprotein complex that regulates the quantity of β-catenin in the cytoplasm by phosphorylation, this interaction causes β-catenin to be degraded by the proteasome in the absence of Wnt. [97]. Figure (1-7) Schematic representation of the Wnt/β-catenin signaling pathway.

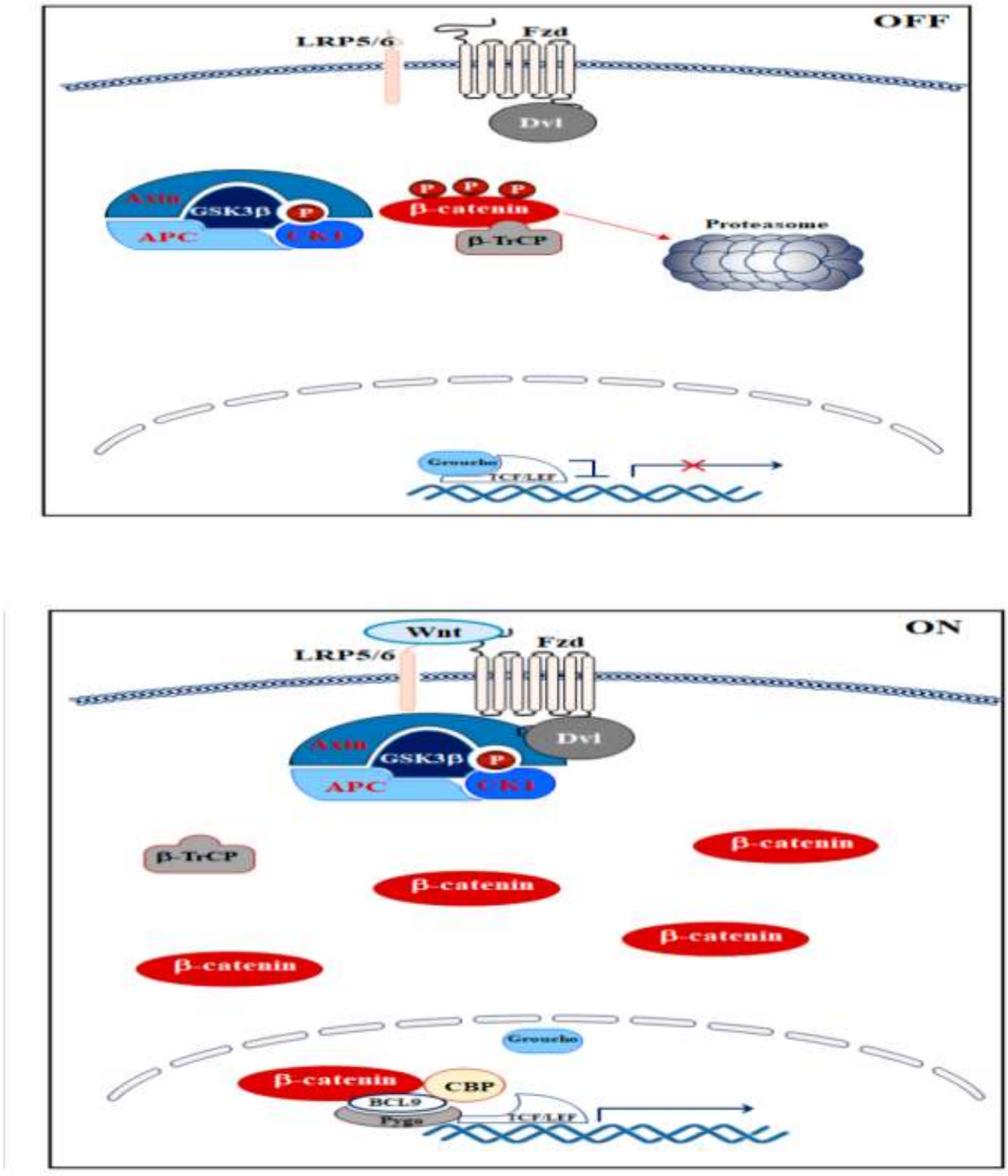


Fig. (1-7): Schematic representation of the Wnt/β-catenin signaling pathway, which is inactive in the absence of Wnt ligands (OFF) and active upon binding of Wnt ligands (ON). Arrows show activation while T-bars show inhibition [97].

1.6.4.Association between Canonical Wnt/β-Catenin Pathway and Type 2 Diabetes

β-catenin and Wnt signaling have been linked to the control of metabolism and the development of Type II diabetes. It has been discovered that elements of the Wnt pathway have a role in the growth of beta cells, the metabolism of cholesterol, the release of insulin, and the creation of GLP-1 (glucagon-like peptide-1). TCF4, a transcription factor that interacts to beta-catenin, has also been discovered as a Type II diabetes risk gene [96].

The Canonical Wnt and β-catenin pathway (Wnt) signaling has been played a well-established role in the metabolic syndrome like (T2DM). Recent research showed that Wnt signaling pathway have important role in regulating pancreas development additionally in islet function and insulin production and secretion [103].There are seem to be indirect link between Wnt signaling pathway and type 2 diabetes mellites; that through the classical Wnt signaling pathway have been regulating the transcription of the proglucagon gene that leading to expression GLP-1 [104]. The affection of GLP-1 on pancreatic beta cells have been mediated by the classical Wnt signaling pathway partly [105]. Seven transmembrane cell surface receptors (Frizzled) and co-receptors (LRP5) and beta-catenin, ligand binding to both the Frizzled and LRP coreceptor that lead to activate GSK-3 [104]. as well as lead to coactivate beta-catenin transcription factors (T-Cell Factor, TCF) and regulate the downstream proglucagon gene (GCG) all of those resulting to GLP-1 expression . The effectors of the Wnt signaling pathway on 1- low-density lipoprotein receptor-related protein 5 (LRP5), 2- transcription factor 7-like 2 (TCF7L2), and 3- the downstream gene glucagon (GCG). LRP5 have important role in glucose and lipid metabolism in animal experimental studie , linked to type 1 diabetes [106]. Also TCF7L2 have to be the strongest candidate associated gene with T2DM [107]. While GCG have been expressed in intestinal epithelial endocrine

located on the chromosome 2q24.2 and have major role during encoded several proteins major for regulation of proglucagon and glucagon-like peptide (GLP)-1 and (GLP)-2, One of the most important functions of GLP-1 that plays a role in the development and treatment of type2 diabetes Miletus [104].

1.7. Electrolytes

1.7.1. Calcium Ion

Calcium ions (Ca^{2+}) are released from bone matrix during osteoclast-mediated bone resorption [108]. 99% of calcium (the most common mineral in the human body) is consumed by the skeletal system, while the remaining 1% moves between intracellular reserves and extracellular fluid and takes part in vital biological processes such forming the signaling transduction system and regulating cellular functioning. About half of the calcium in blood that is circulating is attached to proteins, while the other half is in a bioactive ionized state [109].

Calcium ions (Ca^{2+}) are one of the essential elements for biological growth, but the abnormal concentration of Ca^{2+} can also lead to various diseases [110].

Blood calcium level is tightly regulated, with a normal range of serum calcium concentration between 8.6 and 10.3 mg/dL or 2.2 to 2.6 mmol/L. Major hormones participating in the regulation of calcium homeostasis are parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D(1,25$(OH)_2$D) [108] Calcium (Ca)ion is a versatile intracellular messenger that is used throughout the life cycle of an organism to control diverse biological processes. It has been suggested that diabetes and cardiovascular disease are linked by a common defect of divalent cation metabolism, including calcium ion [111].

An increase in calcium ion is necessary for insulin production since it is a biological function that depends on calcium ion. The release of calcium from

bone tissue may be the cause of this rise. With increased urine excretion, blood calcium levels in children with diabetes decline. Patients and increased cytosolic free calcium were both noted. Type 2 diabetics have lower extracellular ionized calcium levels [39].

Insulin secretion and action include calcium. The development of diabetes, especially type 2 diabetes, and altered calcium homeostasis are related (T2D) [109].

Plasma calcium plays an important role in insulin secretion in these cells. Therefore, if extracellular calcium ion is removed or decreased insulin secretion is also blocked or reduced. A potentially important role for calcium ion status in the development of diabetes is suggested since calcium ion intake was found to be lower in patients with diabetes compared to controls, Low calcium ion intake found to be inversely associated with incident type 2 diabetes [39]. There is mounting evidence that altered calcium ion homeostasis may play a role in the development of type 2 diabetes; calcium ion has been suspected as a diabetes risk modifier in animal studies. Calcium ion changes in primary insulin target tissues may play a role in peripheral insulin resistance. Calcium ion deficiency may have a negative impact on glucose levels; calcium ion supplementation may be beneficial in optimizing glucose metabolism. [112].

Ca^{2+} influx into the cytoplasm is a result of oxidative stress. Ca^{2+} influx into mitochondria and nuclei is caused by an increase in cytoplasmic Ca^{2+} concentration. Ca^{2+} speeds up and disrupts normal metabolism in mitochondria, which kills cells. Ca^{2+} influences gene transcription and the nucleases that regulate cell death in nuclei. Ca^{2+} can control the phosphorylation and dephosphorylation of proteins in nucleus and cytoplasm, which in turn can modify the signal transduction pathway [113].

1.7.2. Sodium Ion

Sodium ion is one of the most crucial electrolytes in the extracellular fluid because it is an osmotically active cation. It controls the modulation of the membrane potential of cells as well as the amount of extracellular fluid. As part of active transport, sodium and potassium are transferred across cell membranes. The kidneys regulate sodium levels. The bulk of sodium reabsorption occurs in the proximal tubule. Sodium is reabsorption in the distal convoluted tubule. Aldosterone-activated sodium-chloride symporters, which transport sodium, are regulated by this hormone [114]. The sodium ion is thought to be crucial for maintaining the pressure of osmotic fluid. Ionized Na^+ is crucial for β-cell activity and insulin release regulation. The binding of an agonist or antagonist to the 2-receptor and the inhibition of insulin release are both significantly influenced by Na ion. The part sodium ion stimulation plays in pancreatic beta-cell secretion a rise in sodium ion membrane permeability for the generation of action potential in excitable cells. About 50% of the sodium in the body is found in extracellular fluid, 45% is found in bones, and the remaining percentage is found inside cells. When serum levels are low, bone crystals act as a reservoir and release sodium[115]. Human serum sodium levels normally range from 135 to 145 mEq/L. Increases in the ratio of total water to sodium, or hyponatremia, are observed in a number of medical disorders, including congestive heart failure and liver illness. Hyponatremia is defined as having a sodium concentration of less than 135 mEq/L. On the other hand, a high salt diet, dehydration, congestive heart failure, Cushing's disease, liver failure, osmotic diuresis (related to hyperglycemia), central diabetes insipidus (DI), or nephrogenic DI can also cause a rise in sodium levels [116].

1.7.3. Potassium Ion

Potassium ion is a chief cation in the intracellular fluid and plays a critical role in water- electrolyte balance [117]. Potassium is crucial for maintaining cell membrane electrical potential, a deviation from normokalaemia induces electrophysiological perturbations with negative effects on cardiac electrophysiology [118]. Potassium is the principal intracellular action and maintenance of the distribution of potassium between the intracellular and the extracellular compartments relies on several homeostatic mechanisms; when these mechanisms are perturbed, hypokalemia or hyperkalemia may occur [113]. Hyperkalemia (HK) is defined as a serum potassium concentration above the upper limit of the normal range (3.5 to 5.5 mmol/L) [118]. Hyperkalemia with high potassium levels, may lead to cardiac arrhythmias and death. It was suggested decades ago that there may be an elevated risk of hyperkalemia in people with diabetes , and hyperkalemia has been associated with diabetic nephropathy [119]. Several chronic diseases are known to increase the risk of HK; most notably, advanced chronic kidney disease (CKD), chronic heart failure (HF), type 2 diabetes (T2DM) and hypertension [78].

A plasmatic potassium (K^+) content below 3.5 mEq/L is considered hypokalemic. In those over 55 year with diabetes, the incidence of hypokalemia ranges from 1.0 to 1.2%. This frequency rises with age and is considerably greater in people with concomitant CKD. The use of diuretics is one of the major risk factors for hypokalemia; 10 to 50% of individuals on this type of medicine may experience this condition. (Potassium problems, either hypokalemia or hyperkalemia,) have been linked to an increase in all-cause mortality among diabetic people, particularly in those who also have accompanying comorbidities including chronic renal disease and heart failure [120].

1.7.4. The Roles of Potassium and Calcium Ions on the Mechanism of Insulin Secretion[121].

Glucose crosses through the transporter Glu2 on the membrane of beta cells (which is opened when it's stimulated by high concentrations of glucose in the blood) glucose enters the beta cells of the pancreatic organelle and is glycolysis in the cytoplasm and is completely oxidized in the mitochondria to give 38 ATP, which was stimulates the opening of the potassium channel (transit of potassium from the blood into the cytoplasm of beta cells) Thus, the concentration of the positive charge increases in the interior of the beta cell, which stimulates the opening of the calcium ion channel into the cytoplasm of the beta cell, which in turn presses on the sac containing the insulin hormone by releasing and crossing through the insulin transporter into the bloodstream, as in scheme (1-8) the scheme was drawing according to the scientific base taken from the references.

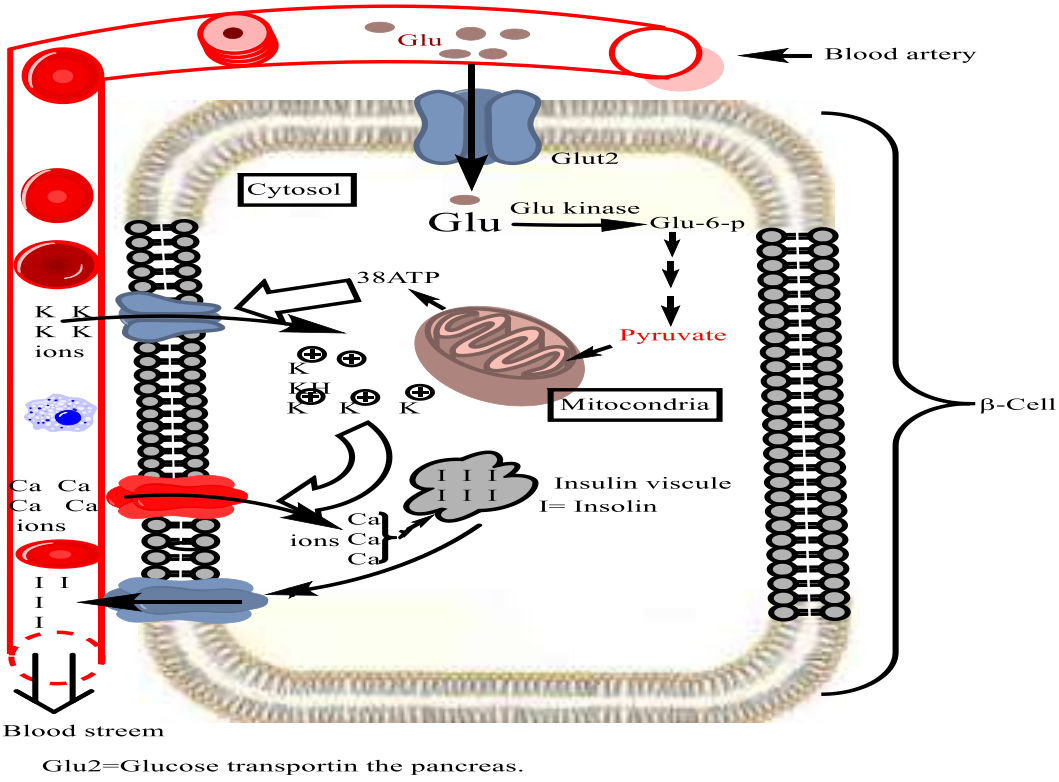


Fig. (1-8): The diagram shows the relationship of the secretion of the hormone insulin in the blood with concentrations of potassium and calcium.

Aims of the Study

To investigate the relationship between butyrylcholinesterase and beta-catenin in sera of patients with Diabetes mellitus Type 2, which are required to estimation the levels of the following parameters related with.

1- Estimation the levels of antioxidant enzymes (SOD, GPx, and Lipoic acid synthetase).
2- Estimation the levels of some non-enzymatic antioxidants (lipoic acid).
3- Estimation of the end product of lipid under oxidative stress reaction (MDA).
4- Estimation the levels of some electrolytes (Na, K, Ca).
5- Determination the relationships between these variables with BuChE,and β-Catenin .

Chapter Two

Materials and Methods

2. Methods and Materials

2.1. Materials

2.1.1. Chemicals and Kits

The chemicals used in the study are shown in the table (2-1) below:

Table (2-1) :The Chemicals and Kit

Chemicals and Kit	Chemical formula	Purity%	Company Name
4-Nitro blue tetrazolium chloride (NBT)	$C_{40}OH_3ON_{10}OCL_2$	99%	Fluka
Riboflavin	$C_{17}H_{20}N_4O_6$	98%	Fluka
Triton-x100	$C_{14}H_{22}O(C_2H_4O)n$	D:1.06	BDH,England
Sodium cyanide	NaCN	95%	Sigma-Aldrich
L-methionine	S2NO11H5C	98%	Sigma-Aldrich
Hydrochloric acid	HCL	39%	Fluka
Sodium hydroxide	NaOH	99%	Aldrich
Di Potassium hydrogen ortho phosphate	K_2HPO_4	98%	Fluka
Trichloroacetic AcidTCA	$C_2HCl_3O_2$	98%	Fluka
Thiobarbutiric Acid TBA	$C_4H_4\ N_2O_2S$	98%	Fluka
Human β-Catenin ,β-Cat ELISA Kit	-	-	Bioassay Technology Laboratory(China)
Human, Butyrylcholinesterase (BCHE)ELISA Kit	-	-	Sunlong Biotech (China)
Human Lipoic acid synthetase (LIAS) ELISA Kit	-	-	Sunlong Biotech (China)
Human Glutathione peroxidase,GPX ELISA Kit	-	-	Bioassay Technology Laboratory(China)
Potassium determination Kit	-	-	Human(Germany)

Sodium determination Kit	-	-	Human (Germany)
Calcium deremination Kit	-	-	Human (Germany)
Glucose kit			Human(German)
Alpha-Lipoic acid pure powder	$C_8H_{14}O_2S_2$	99%	Sigma
Methanol	CH_3OH	99%	Sigma
Chloroform	$CHCl_3$	99%	Sigma
Disodium hydrogen phosphate	Na_2HPO_4	99%	Merck
Acetonitrile	CH_3CN	99%	Sigma

2.1.2. Laboratory Equipment:

Table (2-2) : The instruments used in the research

Instruments	Company Name
Centrifuge	Hermle -Germany
Water bath	BS-11 Korea
Spectroscan 60DV	Management co. ltd (U.K.)
pH meter	Inolab ph7110 Germany
Vortex meter(VM-2000)	Karl Kolb Germany
Oven	Memmert Germany
Sensitive Electronic Balance	Stanton 461 AN (Germany)
autoclave	japan
Elisa reader PKL	Italy
Elisa microplate reader (800TS)	USA
UV-VIS Spectrophotometer UV-1900 i	japan
Cobas c111	Rosh- Germany
HPLC system	Knauer- Germany

2.2 . Study design

The study design is case-control study conducted at the Center for Diabetes and Endocrinology in Iraq , Babylon City, Marjan Teaching Hospital. Included in the following criteria is both patients and healthy people: The age (30 to 60) years of type 2 DM who visited diabetes centers during the study period from December 2021 to March 2022.

2.2.1. Study groups

A. Patients group

This group in the current study included 60 patients with type 2 diabetes who were diagnosed by a consultant physician based on clinical symptoms and laboratory results (FBS, HBA1c). HBA1c The concentration of glycated hemoglobin in the blood was measured in the main laboratory inside Marjan Hospital using the Cobas C111.

B. Control Group

This group included 30 volunteers; their ages ranged from 30-60 years

Note: - The control group measured FBS and HbA1c.

Inclusion criteria: Patients with type 2 diabetes who were not taking insulin were chosen, and controls were selected if their FBS and HbA1C levels were normal.

Exclusion criteria: Relative subjects are excluded as exclusion criterion. Furthermore, every patient who has T1DM, any type of cancer or tumor, any apparently acute inflammation, heart diseases, hypertension, chronic liver disease, Smokers, Pregnant and obese.

2.2.2. Collection of Blood samples

After at least (8-10) hours of fasting, blood was drawn via vein puncture with plastic disposable syringes, with each healthy control and patient receiving up to 5mL of venous blood. The blood is inserted into the gel tube, which is then left at room temperature for 30 minutes to allow the clotting process to begin. After that, the samples were centrifuged at 600 xg for (15) minutes to separate the serum. The sera were divided into aliquots and stored in aliquots (250µl) in 5 Eppendrof tubes in a freezer at (-65 ° C) until use.

2.2.3. Body mass Index (BMI) [122].

Body mass index was determined in all participants based on a weight-to-height ratio produced by using a mathematical equation in which the weight in kg is divided by the square height in meter, and the results were analyzed.

were taken into account as follows:

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

Underweight $\leq 18.5(kg/m^2)$

Normal weight between 18.5 - 24.9(kg/m^2)

Overweight between 25-29.9(kg/m^2)

Obese $\geq 30(kg/m^2)$

2.3. Methods

2.3.1. Determination of Fasting Blood Glucose Concentration

2.3.1.1.Principle

The level of glucose in the blood serum was determined by the enzymatic colorimetric method ,using the diagnostic kit, where the interaction principle of this method depends on Enzymatic oxidation of glucose by the enzyme Glucose oxidase (GOD) and in the presence of oxygen to gluconic acid and hydrogen peroxide, which in turn reacts with phenol and 4-aminophenazone in the presence of the enzyme peroxidase (POD). To produce a pink complex whose intensity is proportional to the glucose concentration in the sample.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} H_2O_2 + \text{Gluconate}$$

$$2H_2O_2 + \text{Phenol} + 4\ \text{Amino-antipyrine} \xrightarrow{\text{Peroxidas}} 4H_2O + \text{Quinonimine}$$

2.3.1.2. Reagents

1- Reagent 1 (Buffer): Consist of 150 mmol/L of phosphate buffer pH7.5 and 2 mmol/L Phenol

2-Reagent 2 (Enzymes): Consist of ≥20 000U/L of glucose oxidase (GOD), ≥1000 U/L of peroxidase and 0.8mmol/L of 4-amino antipyrine (PAP).

3- Reagent 3 (Standard): Consist of 100 mg/dL .

2.3.1.3.Preparation of Reagents

Working reagents are made by combining the material in the vial containing reagent 2 (enzymes) with the vial of reagent 1 containing reagent 2 (Buffer). The mixture is stirred to finish the dissolution of all components softly. The technique is described in the table below:

Reagent	Blank	Standard	Sample
Working reagent	**1000µl**	**1000µl**	**1000µl**
Standard (STD)		**10µl**	
sample			**10µl**
Distilled water	**10µl**		

After allowing the tube to stand at 37 ºC for 10 minutes, the absorbance was measured at 500 nm. Against reagent blank.

2.3.1.4. Calculation

Glucose (mmoL/L) $= \frac{Abs.(sample)}{AbS(.standard)} \times Stand.Conc.$

Conc. of standard =5.55 mmoL/L

Reference Values:(4.6-6.4)mmol/L

2.3.2 Estimation of Serum Levels of Human β-Catenin

2.3.2.1 Principle:

The Elisa kit used sandwich Elisa technique to quantitative determination of Human β -Catenin (known as β-Cat) in serum. The plate was pre-coated with human β-Catenin antibody. The tested sample was introduced to the wells to catch antibodies that had been coated on them. Then biotinylated anti human β - Catatenin antibody were added to binds to

β -Catenin in the tested sample .Then Streptavidin-HRP was added and binds to the Biotinylated β -Catenin antibody. Unbound Streptavidin-HRP was extracted by washing during the washing step after incubation of the plate. Then, substrate solution was added, the color develops according to the amount of human(β-catenin .The reaction ends by adding acidic stope solution and the absorbance was determined at 450 nm.

Table (2-3): Kit component of human β-Catenin bioassay

Components	Quantity
Standard Solution :For β -catenin (80ng/ml)	**(0.5ml) x1**
Pre-coated Elisa Plates	**12 * 8 well strips x1**
Standard diluent	**(6ml) x1**
Strepta-vidin-H.RP	**(6ml) x1**
Stop Solution	**(6ml) x1**
Solution of substrate A.	**(6ml) x1**
Solutionof substrate B.	**(6ml) x1**
Wash Buffer Concentrating (25x)	**(20ml) x1**
Biotinylated Human β -Cat	**(1ml) x1**
Plate Sealer	**2 pics**

2.3.2.2 Reagent Preparation

1. The reagents placed in room temperature before using.

2.Standard Reconstitute the 120µl of the standard (80ng/ml) with 120µ of standard diluent for preparation (40 ng) standard stock solution. Gentle agitation was done for the standard for 15 mins before dilutions have done. Serial dilution from the standard stock solution(40 ng/ml) were done to prepare duplicate standard by1:1 using standard diluents for production of

20ng/ml,10ng/ml,5ng/ml and 2.5ng/ml solution for β-Catenin. Standard diluent was zero standard (0 ng/ml).The Serial standard dilution shown in Table (2-4) and figure(2-1) . The remaining solution was kept in deep freeze at -20° C and was used confines of one month.

Table (2-4): Serial standard dilution of β-Catenin

Standard Concentration	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
80ng/ml	40ng/ml	20ng/ml	10ng/ml	5ng/ml	2.5ng/ml

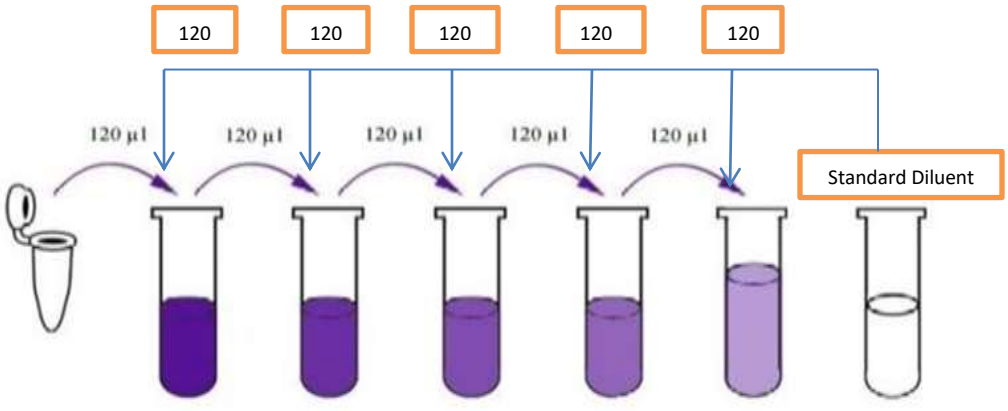


Fig (2-1): Serial standard dilution of β-Catenin

3. Wash Buffer: 20 ml of washing buffer solution (25x) was diluted together with de-ionized or distilled water until 500ml of 1x washing buffer is form.

2.3.2.3 Assay Procedure

1. The standard solutions and samples were prepared according to the instructions. The experiment was performed at room temperature.

2. The number of strips required for the assay should be determined. Also, the unused strips were stored at (2-8) °C while the others were placed into the frame.

3. The standard wells were filled with 50µl of standards solution .

4. Forty µl samples was placed in the wells of sample then (10µl) of Anti-β-Catenin antibodies was placed in sample wells, then 50 µl of streptavidin-HRP added in standard wells and sample wells .(Not blank control well) thoroughly mixing the reagents The plate was then covered. and incubated for 60 minutes at 37 degrees Celsius.

5. The plates were washed (5) times using washing buffer well was soaked with (300µl) washing buffer for 30 seconds -1minute for every washed. The Plates were blotted dry using papers towels.

6. Fifty µl of substrate solutions A was added into each well then (50µl) substrate solution B was added to every well. After covering the plates, incubate them in the dark for 10 minutes at 37°C.

7. Fifty µl of stopped solution added in every well, the color changing instantly from blue to yellowish.

8. Within 10 minutes of the stop solution being added, the optical density for each well was determined using the microplate reader at 450 nm.

9. dose response standard curve is used to evaluate the concentration of β-Catenin in serum as shown in figure(2-2).

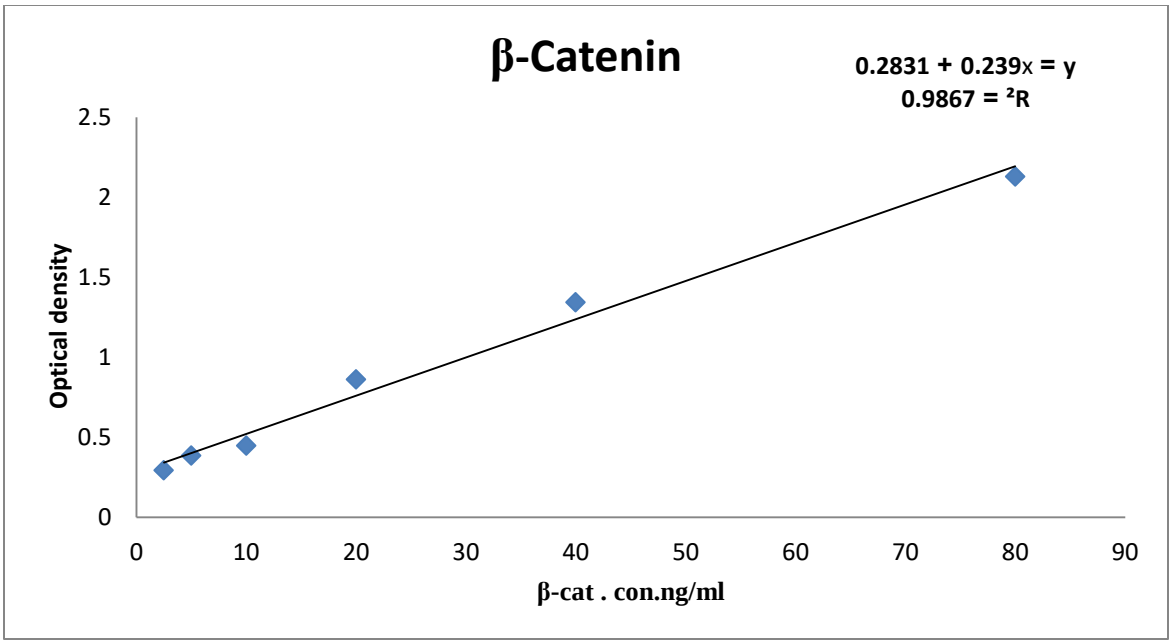


Fig. (2-2): Standard curve of Human β-Catenin

2.3.3. Estimation of Serum Levels of Butyrylcholinesterase (BuChE)

2.3.3.1. Principle

ELISA kit used Sandwich ELISA as the method. The Micro ELISA strip plate has been provided, in this kit was pre-coated with an antibody as specific to BuChE. Standards or samples had been added to the appropriate Micro ELISA strip plate wells and combined to the specific antibody. Then a Horseradish Peroxidase (HRP)-conjugated antibody specific for BuChE are added to each Micro ELISA strip plate well and incubated. Free components are washed away. The Tetramethylbenzidine (TMB) substrate solution had been added to each well. Only those wells that contain (BuchE and HRP) conjugated BuchE antibody that would be appear blue in color and then turned into yellow after addition of the stop solution. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm. The

optical density value was proportional to the concentration of BuchE. By comparing the optical density of the samples to the standard curve, you may determine the concentration of BuchE in the samples.

Table (2-5): Kit component of human Butyrylcolinesterase bioassay:

Components	Quantity	Storage
User manual	1	R.T.
Closure plate's membrane	2	R.T.
Sealing Bags	1	R.T.
Micro Elisa- stripplate	1	2-8ºC
Standard: 270 pg/ml	(0.5ml×1 bottle)	2-8ºC
Standards diluents	(1.5)ml×1 (bottle)	2-8ºC
H.R.P. –Conjugate reagent	(6 ml×1 bottle)	2-8ºC
Samples diluent	(6 ml×1 bottle)	2-8ºC
Chromogen- Solutions A	(6 ml×1 bottle)	2-8ºC
Chromogen- Solution B	(6 ml×1 bottle)	2-8ºC
Stop Solution	(6.ml×1 bottle)	
Washing solution	20ml 30X×1bottle	2-8ºC

2.3.3.2. Assay procedure

1. Standards dilutions for (BuchE) were (180 pg/mL), (120 pg/mL), (60 pg/mL), (30 pg/mL) and (15pg/mL). The Serial standard dilution shown in Table (2-6).

2. The blank control was left in an empty well on the Micro-Elisa strip-plate. 40µl Sample dilution was added to the wells of the samples. The "dilution factor was 5"time was done on a buffer as well as a 10 µl sample.

3. After sealing the samples with the "Closure plate membrane," they were incubated at 37°C for thirty minutes.

Table (2-6) :Serial standard dilution of Btyrylcholinesterase

180pg/ml (BuchE)	Standard No.1	300µL Original Standard +150µL Standard diluent
120pg/ml (BuchE)	Standard No.2	300µL Standard No.1 + 150µL Standard diluent
60pg/ml (BuchE)	Standard No.3	150µL Standard No.2 + 150µL Standard diluent
30pg/ml (BuchE)	Standard No.4	150µL Standard No.3 + 150µL Standard diluent
15pg/ml (BuchE)	Standard No.5	150µL Standard No.4 + 150µL Standard diluent

4. The concentrated washing buffer was diluted thirty times with distilled water.

5. The "Closure plate membrane" was gently peeled off, followed by aspiration and refilling with the wash solution. The wash solution was removed after 30 seconds of relaxation. This washing step was repeated for (5) times.

6. Added fifty µl "HRP Conjugate reagent" to all wells except the blank control well.

7. Incubated was carried out, as described in step three

8. The washing procedure was performed as described in step 5.

9. The coloring: 50 µl of Chromogenic Solution A and 50 µl of Chromogenic Solution B were added to each well, light shaking was conducted for well mixing, and the samples were incubated at 37°C. fifteen minutes Light should be avoided throughout the coloring procedure.

10. Termination: To end the reaction, add 50 µl of stop solution to each well, The color of the well has been altered from blue to yellowish..

11. Each well's optical density was measured at 450nm (within 10 minutes of adding stop solution). dose response standard curve is used to evaluate the concentration of BuChE in serum as shown in figure(2-3).

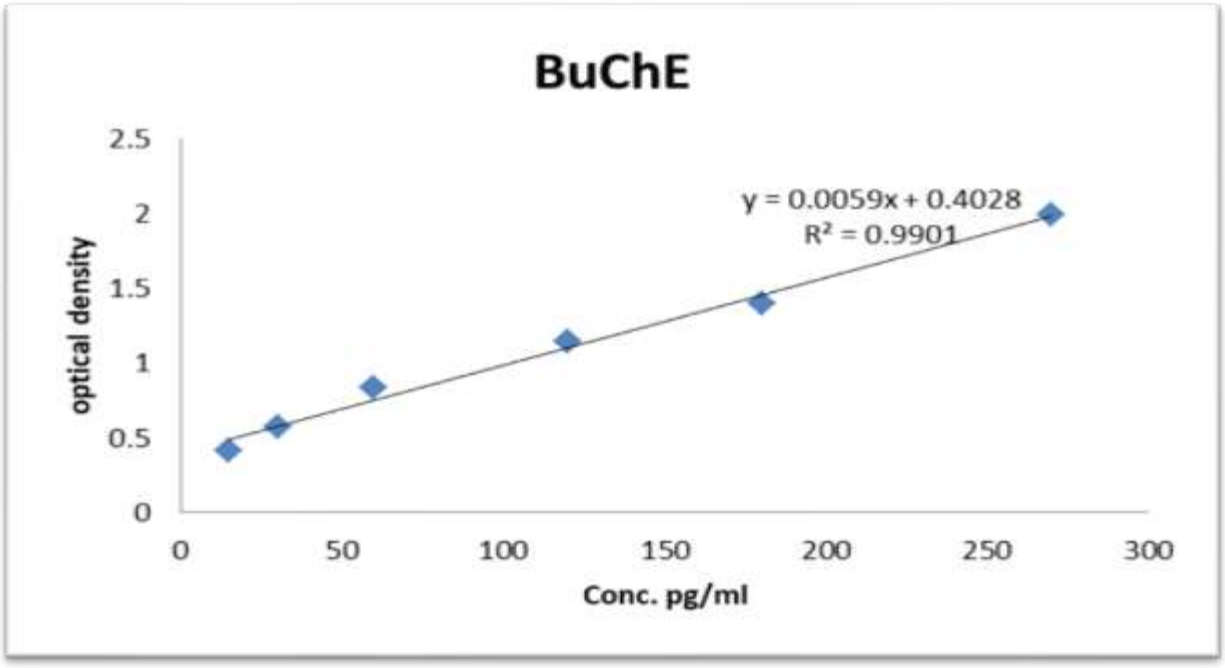


Figure (2-3) Standard curve of Human Butyrylcholinesterase (BuChE)

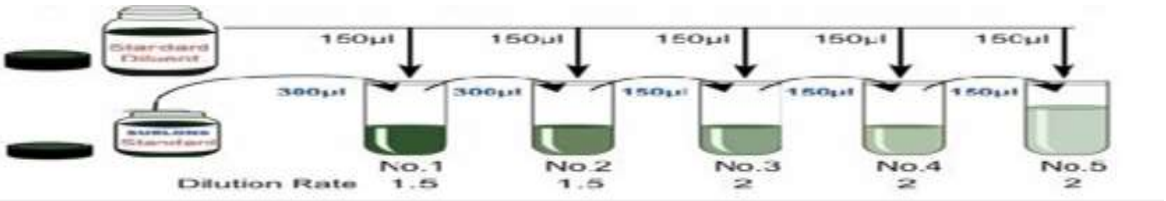
2.3.4. Estimation of Serum Levels of Human Lipoic Acid Synthetase

2.3.4.1. Principle

The Sandwich ELISA technique had been used in this ELISA kit. The Microelisa stripplate included in this kit was precoated with ELIAS specific antibody. Standards was mixed with the particular antibody in the corresponding Micro Elisa strip plate wells. After that in each Micro Elisa strip plate well a Horseradish Peroxidase (HRP) conjugated antibody specific for LIAS is applied and incubated. Free substances were washed away. Each well had been filled with the Tetramethylbenzidine (TMB) substrate solution. Only the wells containing (LIAS and HRP conjugated LIAS antibody) would be showed blue and then turned into yellow once the stop solution is added. At 450 nm, the optical density (OD) had been determined spectrophotometrically. The OD value was proportional to LIAS concentration. Determination of the concentration of LIAS in the samples by comparing the OD of the samples to the standard curve.

2.3.4.2. Assay Procedure

1. Standards dilutions for LIAS were (135pg/ml), (90pg/ml), (60pg/ml), (30pg/ml),(15pg/ml),(7.5pg/ml). The Serial standard dilution shown in figure(2-4) and table (2-7).



Fig(2-4) Serial standard dilution of Lipoic acid synthetase

Table (2-7): Serial standard dilution of Lipoic acid synthetase

90 pg/ml (LIAS)	**StandarD No.1**	**300µL Original Standard +150µL Standard diluent**
60pg/ml (LIAS)	**Standard No.2**	**300µL Standard No.1 + 150µL Standard diluent**
30pg/ml (LIAS)	**Standard No.3**	**150µL Standard No.2 + 150µL Standard diluent**
15pg/ml (LIAS)	**Standard No.4**	**150µL Standard No.3 + 150µL Standard diluent**
7.5pg/ml (LIAS)	**Standard No.5**	**150µL Standard No.4 + 150µL Standard diluent**

2. Sample dilution:

1. Ten µl sample were mixed with forty µl Sample dilution buffer (dilution factor is 5).

2. Then added in sample wells, without touching, with gentle shaking.

3. The plate was then sealed with a Closure plate membrane and incubated for 30 minutes at 37 °C.

4.Dilution: The concentrated washing buffer was diluted thirty times with distilled water.

5. The Closure plate membrane was gently peeled off, and the plate content was removed, before the plate was washed 5 times for 30 seconds with Wash Buffer.

6. Except for the blank control well, each well received 50 µl of HRP-Conjugate enzyme.

7. The incubation period at the same described in Step 3.

8. The washing at the same described in Step 5.

9. Coloring: Fifty µl Chromogenic Solution A and 50 µl Chromogenic Solution B were added, in each well, with gently shaking and then plates were covered and incubated at 37°C in dark within 15 minutes.

10. Termination: In each well, 50 µl Stop Solution was added and carefully mixed. The color transitioned from blue to yellow.

11.The optical density(O.D) was read using Microplate Reader at wavelength 450 nm. dose response standard curve is used to evaluate the concentration of LIAS in serum as shown in figure(2-5).

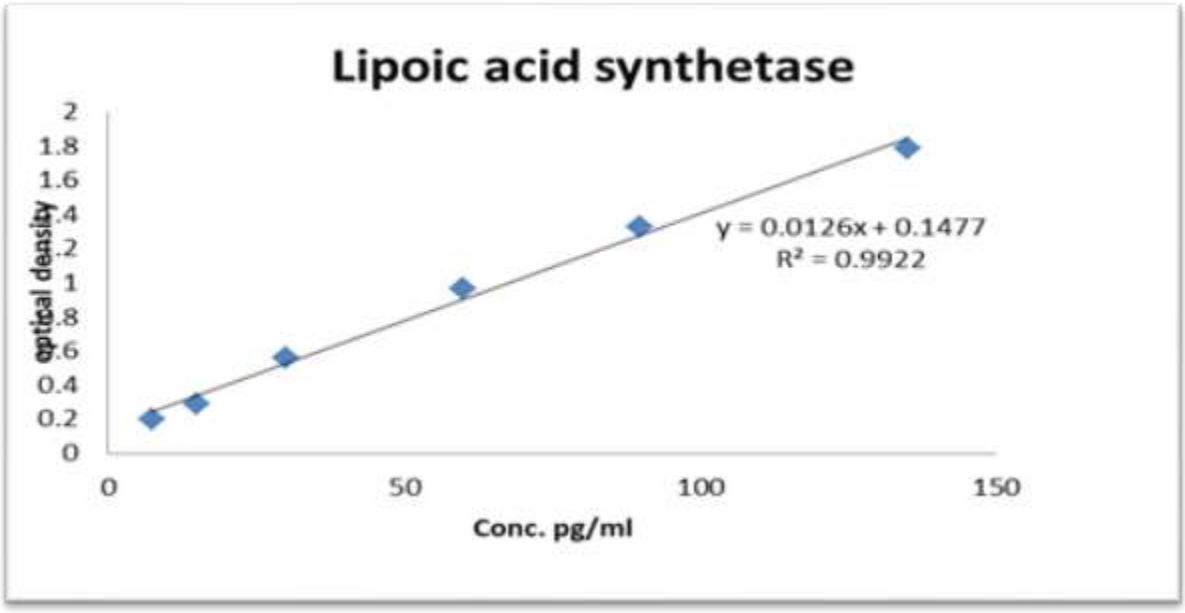


Figure (2-5) :Standard curve of Human Lipoic acid Synthetase

2.3.5. Estimation of Serum Glutathione Peroxidase Activity Level

2.3.5.1. Principle

The sandwich Eliza method was applied in the Eliza kit to assay Human Glutathione Peroxidase (GPX) in serum. The plate was pre-coated with human GPX antibodies. The tested samples were placed in the wells to catch

antibodies coated on them. The biotinylated anti human GPX antibody was then added to the tested sample to bind to GPX. Streptavidin-HRP was then added, which binds to the biotinylated GPX antibody. Unbound Streptavidin-HRP was removed from the plate during the washing stage following incubation. The substrate solution was then added, and the color developed in accordance with human GPX. The reaction ends by adding an acidic stope solution, and the absorb was measured at 450 nm.

2.3.5.2 Reagent Preparation

1. All reagents were placed at room temperature before use

2. Standard Reconstitute 120 µl of the standard (640U/ml) with 120 µl of standard diluent for production of (320U/ml) standard stock solution. Before dilutions, the standard was gently agitated for 15 minutes. Serial dilutions from the standard stock solution (320U/ml) were performed to generate duplicate standards by 1:1 using standard diluents for the preparation of 160U/ml,80U/ml,40U/ml, and 20U/ml solutions. The standard diluent was 0 U/ml.

Table (2-8):Serial standard dilution of Glutathione peroxidase

Standard Concentration	Standard 5	Standard 4	Standard 3	Standard 2	Standard 1
640U/ml	320 U/ml	160 U/ml	80 U/ml	40 U/ml	20 U/ml

3. Wash Buffer: 20 mL of washing buffer solution (25x) was diluted with deionized or distilled water until 500mL of 1x washing buffer was formed.

2.3.5.3 Assay Procedure

1.The standard solutions and samples were produced in accordance with the instructions. The experiment was carried out at a temperature.

2. The number of strips required for the experiment should be determined. Also, the unused strips were stored at (2-8) °C while the others were placed into the frame

3. Fifty µl of standards solution were added into the standard wells.

4. Forty µl of sample was placed in sample wells, then 10 µl of anti-GPX antibodies were inserted in sample wells, followed by 50 µl of streptavidin-HRP in standard and sample wells. (Not blank control well) mixing the reagents completely The plate was then covered and incubated at 37°C for 60 minutes.

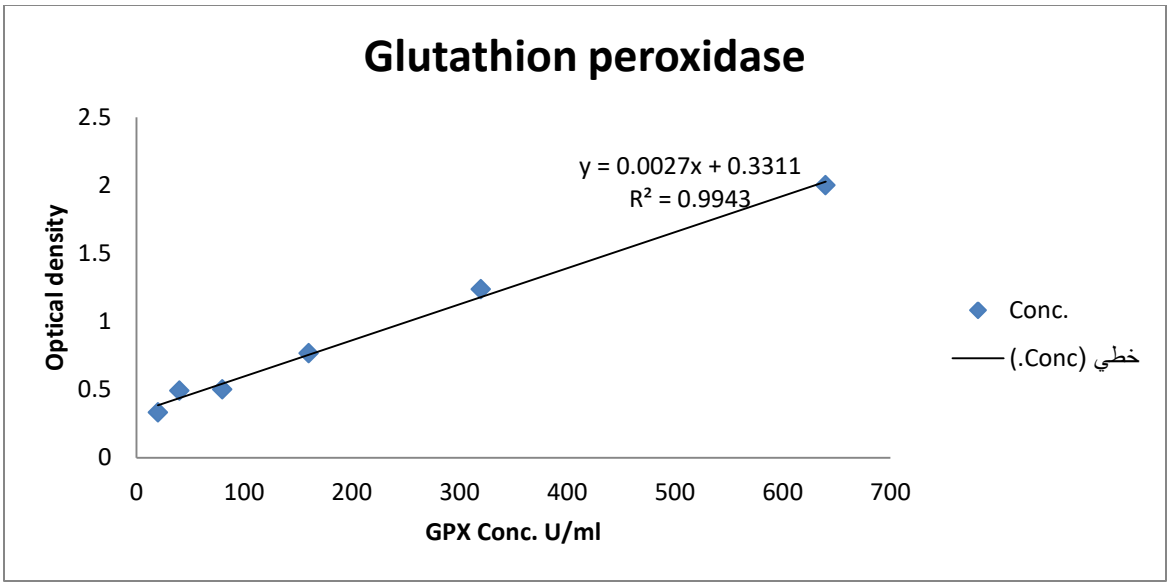
5. The plates were washed five times with washing buffer. Each wash was steeped in (300 µl) washing buffer for 30 seconds to 1 minute. Paper towels were used to wipe the plates dry.

6. Each well added 50 µl of substrate solution A, followed by 50 µl of substrate solution B. After covering the plates, incubate them in the dark for 10 minutes at 37°C.

7. Fifty µl of stopped solution was added to each well, and the color rapidly changed from blue to yellowish.

8. The optical density value for each well was measured using the microplate reader at 450 nm within 10 minutes after adding the stop solution.

9. dose response standard curve is used to evaluate the concentration of GPX in serum as shown in figure(2-6).



Figure(2-6):Standard curve of Human Glutathione Peroxidase (GPX)

2.3.6.Determination of Serum Levels Lipoic Acid[123]

2.3.6.1. Principle

Lipoic acid has been playing important role in energy metabolism. It has been an excellent antioxidant acting in both the cell and at plasma membrane levels. The concentrations of α-lipoic acid had been estimated by high performance liquid chromatography (HPLC). This method involved extraction of lipoic acid by using ethanol and chloroform. Disodium hydrogen phosphateNa_2HPO_4 (pH adjusted to 2.7): acetonitrile: methanol they were used as a mobile phase. The separation had been done through using a C18 column (150 mm) and LA contain a carboxyl group it could be detected by using UV detector. detection had been carried out by using UV detection at 201 nm. The detection of lipoic acid was performed by matching retention time and absorbance spectrum of the standards, the concentration was calculated by serial concentrations (0.1-100 ug/ml) of

external standard materials to build calibration curve between concentration and its equivalent peak area .

2.3.6.2. Procedure

A. Preparation of Standard Solution

Stock standard solution of (1mg/ml) of LA had been prepared by dissolving (pure powder) in methanol. The concentrations of LA in the working standards were (0.1,1,10,100) ug/ml. Calibration curve of alpha-Lipoic acid shown in figure(2-7)

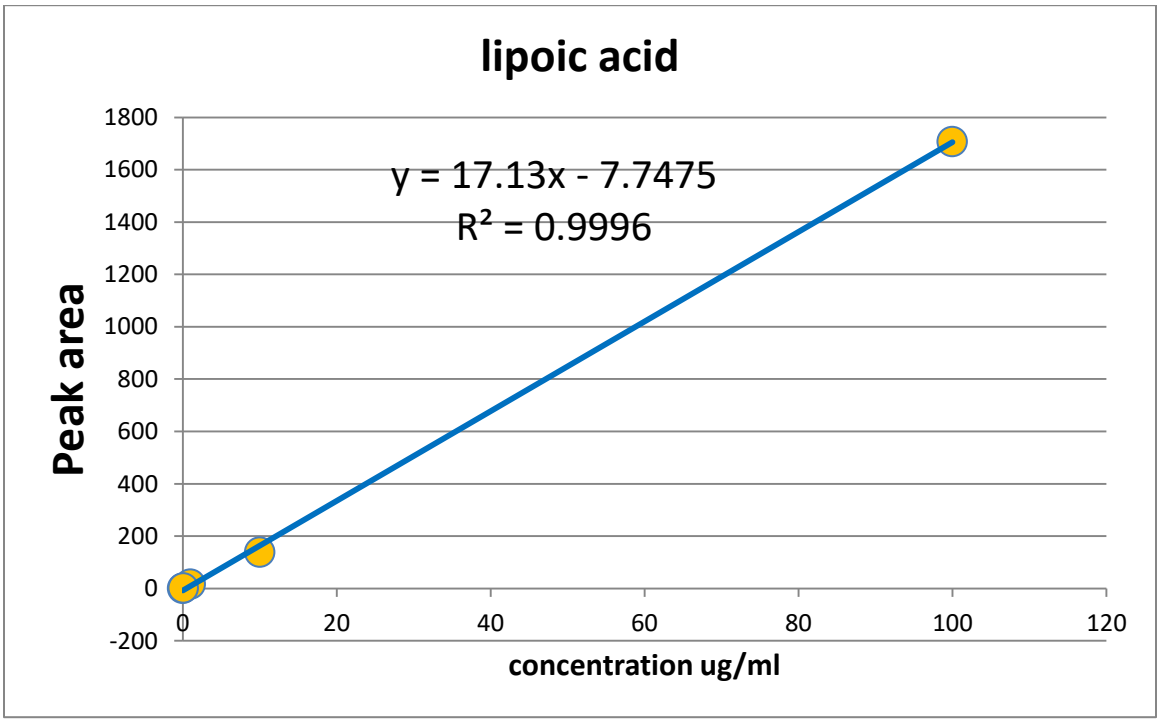


Fig. (2- 7): Calibration curve of alpha-Lipoic acid.

B. Sample Extraction

Three hundred (300) microliters of the serum had been mixed with 300 µl of ethanol and 1.8 ml of chloroform; the mixture had vortex-mixed at high speed for 2 min. After centrifugation for five min at 520 xg and (4 ◦C). Six hundred microliters of the supernatant was separated and evaporated in

nitrogen evaporator for 10 min. The dried residue was reconstituted with 100 µl of the mobile phase and 50 µl of the sample was directly injected into the HPLC column.

C. Mobile Phase

One hundred ml buffer was prepared by taking 705 mg and dissolved in 50 ml distilled water, then completing the volume to 100 ml and equalizing the pH using drops of HCl (1N).

The mobile phase was comprised of 50ml disodium hydrogen phosphate buffer, acetonitrile and methanol in the ratio of 50:30:20. Prior to the preparation of mobile phase, the solvents were degassed separately using a Millipore vacuum pump with 0.45 µm filter paper.

D. Chromatographic Analysis

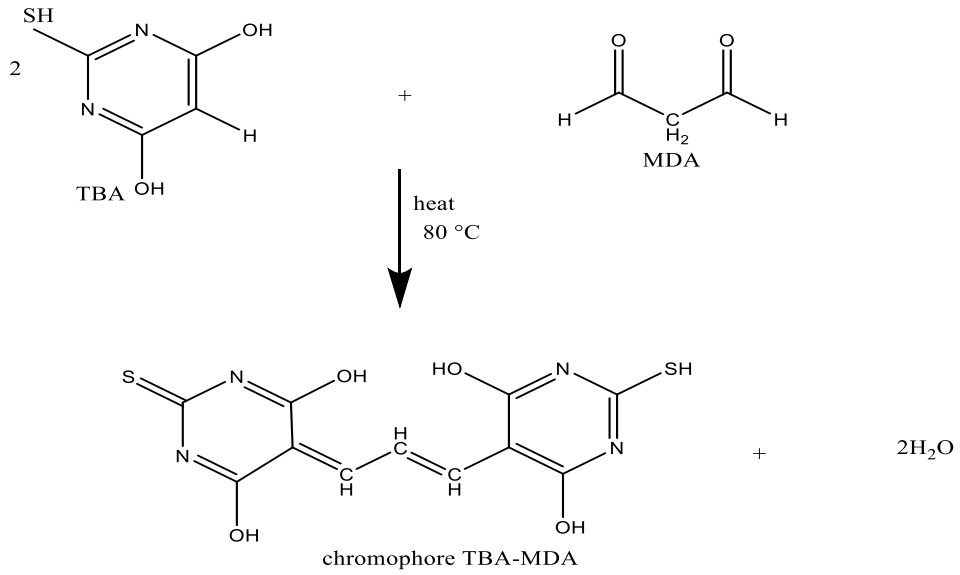
Chromatographic analysis had been performed by using HPLC system that consisted of (2) pumps (LC- 10ATvp) and an auto sampler (SIL-HTA) with built in system controller (SCL-10Avp), class VP-LC workstation had been used for data collection and acquisition. The analytical column had a C18, 250×4.6 mm ID (internal diameter), 5µl particle size was protected by a compatible guard column. The UV detector had been set at 201 nm. The chromatogram was running for five minutes at a flow rate of 1 ml per min, with column oven temperature maintained at 40℃. Unknown concentrations had been derived from linear regression analysis of the peak height of the analyte vs. concentration curve. The linearity was verified using estimates of correlation coefficient (r). The detection of lipoic acid was performed by matching retention time and absorbance spectrum of the standards, the concentration was calculated by serial concentrations (0.1,1,10,100 ug/ml)

of external standard materials to build calibration curve between concentration and its equivalent peak area . The Chromatograms of Lipoic acid of patient with diabetes mellitus type2 and controls shown in figures(1to7in appendix).

2.3.7. Determination of Serum Malondialdehyde Levels

2.3.7.1. Principle:

The basis of (MDA) determination had been based on the spectrophotometric measurement of the color produced during the reaction of thiobarbituric acid (TBA) with MDA, [124]. As shown in Scheme (2-8).



Scheme.(2-8): Reaction between (MDA and TBA)

2.3.7.2. Reagents

1. TBA 0.6 percent, made by dissolving 0.6 g of thiobarbutiric acid in 100 mL of D.W.

2. TCA 17.5 percent was made by dissolving 17.5 g of trichloroacetic acid in 100 mL of D.W.

3. TCA 70%, made by dissolving 70 g of trichloroacetic acid in 100 mL of D.W.

2.3.7.3. Procedure

The addition had been performed by a set of three tubes representing sample, reagent, and blank.

Reagents	Sample	Blank
Serum	150 µL	…….
TCA (17.5 %)	1000 µL	1000 µL
TBA (0.6 %)	1000 µL	1000 µL
All tubes were mixed by vortex, incubated in boiling water bath (80 °C) for 15 minutes and then left to cool (at 25°C)		
TCA (70%)	1000 µL	1000 µL
D.D.W	…….	150 µL

The solution had been allowed stand at room temperature for (20) minutes and centrifuged at (450Xg) for 15 minutes.The absorbance of all tubes had been measured clear supernatant at (532 nm) using spectroscan 60D.

Calculation:

The concentration of MDA µmole/ L = (A sample/L *ε) * D

Where

L = light path (1cm)

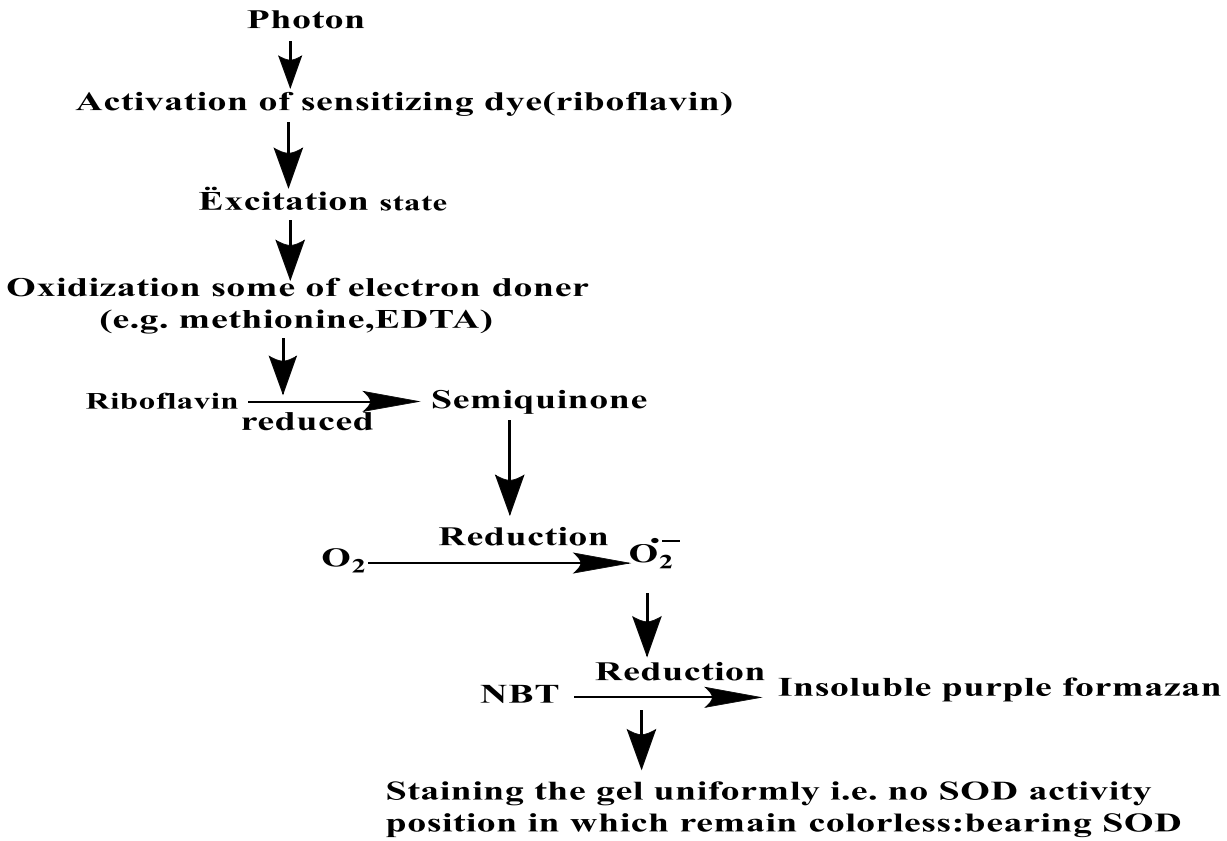
ε = Molar extinction coefficient ($1.56 * 10^5 M^{-1} cm^{-1}$)

D = Dilution factor/ D =1ml (volume used in reference)/0.15ml (volume used in sample)= 1/0.15= 6.7

2.3.8. Determination of the activity of Superoxide dismutase (SOD) in serum.

2.3.8.1. Principle

This depends on the ability of the SOD enzyme to inhibit (Nitro blue tetrazolium) NBT reductase by superoxide, which is formed by the reaction of riboflavin with oxygen in a photochemical reaction, where a purple colored complex is formed. The absorbance was measured at 560 nm [125]. Scheme(2-9) showing how to determine the activity of SOD enzyme by inhibiting NBT.



Scheme (2-9): Showing how to determine the activity of SOD enzyme by inhibiting NBT reductase according to the patent of AL-Zamely 2020 [125].

2.3.8.2. Reagents Using the Method Approved in a Patent (Oda M. Alzamely 2020):

1.Phosphate buffer concentration (0.05M) pH=7.8

This phosphate buffer solution contains EDTA at a concentration of 0.1 mM and Triton x-100(0.025%)

The solution is prepared as follows:

$$pH = pka + \log \frac{[base]}{[acid]}$$

$$7.8 = 7.2 + \log \frac{[base]}{[acid]}$$

$$\log \frac{[base]}{[acid]} = 0.6$$

$$\frac{[base]}{[acid]} = 3.981$$

Concentration $K_2HPO_4 = \frac{3.981}{4.981} \times 0.05$

Concentration $KH_2PO_4 = \frac{1}{4.981} \times 0.05$

Calculate the ionic strength and find the new pKa value:

$$\frac{\Gamma}{2} = \frac{[HPO_4^{-2}](2)^2 + 2[K^+](1)^2 + [H_2PO_4^-] + (1)^2 + [K^+](1)^2}{2}$$

$$= \frac{0.26}{2} = 0.13$$

Referring to the table (5) in the Segel book to find the ΔpKa value that corresponds to the ionic strength value, which is approximately equal to (-0.32)

$$pka1 = pKa + \Delta pKa$$

$$pka1 = 7.2 + (-0.32)$$

$$= 6.88$$

$pH = pka + \log\frac{[base]}{[acid]}$

$7.8=6.88+\log\frac{[base]}{[acid]}$

$\log\frac{[base]}{[acid]}=0.92$

$\frac{[base]}{[acid]}=8.31$

There are two ways to prepare buffer, either by calculating concentrations or by volumes, according to the proportion of each substance, as follows:

A.

concentration $K_2HPO_4 = \frac{8.31}{9.31} \times 0.05=0.045$

concentration $KH_2PO_4=\frac{1}{9.31} \times 0.05=0.0053$

B.

1. 0.05M Di potassium hydrogen orthophosphate(K_2HPO_4) solution was prepared by dissolving 8.709g of (K_2HPO_4) in 1000ml of distilled water.

2. 0.05 M of Potassium dihydrogen orthophosphate KH_2PO_4 acid was prepared by dissolving 6.805 g of KH_2PO_4 in 1000 ml of distilled water. We put the trapped solutions inside the autoclave for sterilization. 2

Conjugate base volume(K_2HPO_4) $=\frac{8.31}{9.31} \times 1000 \longrightarrow$ =892ml

Conjugate acid volume(KH_2PO_4)$=\frac{1}{9.31} \times 1000 \longrightarrow$ 108=ml

These two volumes of the first and second solutions were mixed and the pH was measured and adjusted to 7.8 to add drops of hydrochloric acid (HCl.,0.1N) if it was higher than 7.8 and sodium hydroxide (NaOH,0.1N), if it was less than (7.8).

A concentration of 0.0001 molar of Na_2EDTA was added by dissolving 0.033621 g of Na_2EDTA. in 1000 ml of Phosphite buffer solution Then 0.25 ml of Triton x-100 was added to the phosphite buffer.

3. A solution of L- methionine with a concentration of(0.2 M) was prepared by dissolving(0.3 g) of L- methionine in(10 mL) of distilled water.

4. A 0.0176 M solution of NBT-2HCl was made to dissolve 0.0141 g of NBT-2HCl in 10 ML of distilled water.

5. Triton x-100 (V/V%1) was prepared in distilled water.

6.A solution of Riboflavin with a concentration of (0.000117M)was prepared by dissolving (0.011g) of Riboflavin in 25 ml distilled water.

7. Sodium cyanide (NaCN) solution (0.002 M): dissolve 0.001 g of NaCN in 10 ml distilled water.

8. Reaction mixture solution: This solution was created by combining the volumes of the solutions made in the previous steps.

117 mL of Phosphite buffer solution +1.25 mL of L- methionine solution+1 ml of NBT-2HCl+0.75 mL of Triton x-100 (Total volume 120mL)

2.3.8.3. Procedure:

1. In each test tube , 3 mL of the reaction mixture solution was added , followed by 0.03 mL of sodium cyanide solution .
2. 0.15 mL of serum and 0.525 mL of phosphate-buffered solution were added to each sample tube While the control and standard tubes were added 0.675 mL of phosphate buffer solution.

3. Sodium cyanide(NaCN)(0.03 Ml) was added to all tubes.

4. Riboflavin solution 37.8 ml was added to all tubes.

5. The tubes were mixed well, then the absorbance was read at 560 nm

6. All tubes were placed in a box lined with mirrors containing two fluorescent lamps (25 watts each) for 15 minutes, the dimensions of the box (75x50x20) cm^3 .Figure (2-10) showing the tubes inside the radiation box.

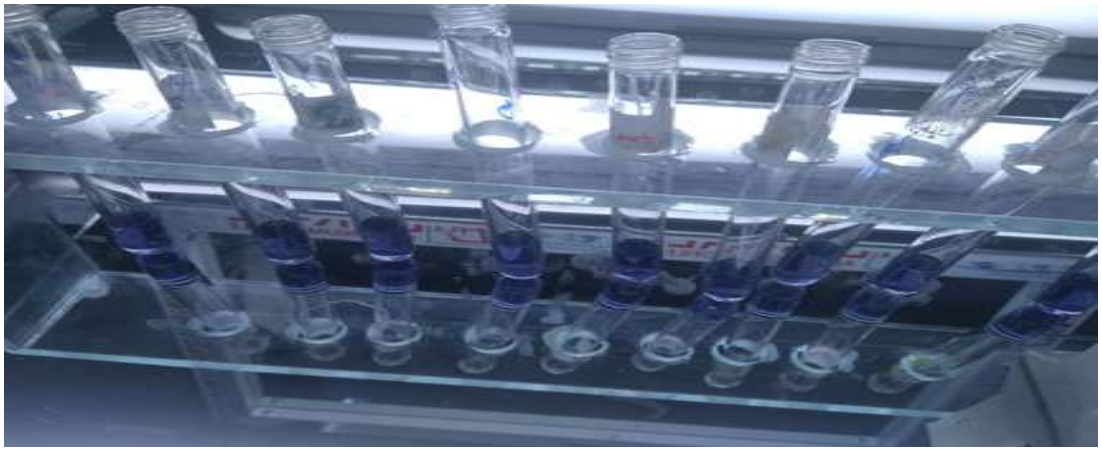


Figure (2-10): Showing the tubes inside the radiation box according to the patent of AL-Zamely & AL-Nemer 2020

7. After the end of the period, the absorbance of all tubes was measured at 560 nm.

8. The activity of SOD was determined by reference to the difference in absorbance (optical density) between absorbances measured before and after placing the tubes in the box.

Reagents	Control	Test	Blank
Reaction mixture solution.	3000 µl	3000 µl	3000 µl
Sodium cyanide solution.	30 µl	30 µl	30 µl
Sample	-	150 µl	-
phosphate buffer solution	675 µl	525 µl	675 µl
Riboflavin solution	37.8 µl	37.8 µl	37.8 µl

2.3.8.4. Calculation of inhibition percentage of SOD:

1. The following relationship was used to calculate the rate of enzyme inhibition:

Inhibition% of sample $= \frac{\Delta C - \Delta T}{\Delta C} \times 100$

ΔC =Absorbance of control after illumination- Absorbance of control before illumination.

ΔT = Absorbance of sample after illumination- Absorbance of sample before illumination.

2. A calibration curve for SOD enzyme inhibition was drawn between the inhibition ratio on the y-axis and the absorbance for different serum volumes on the x-axis for a healthy person as in the figure below.

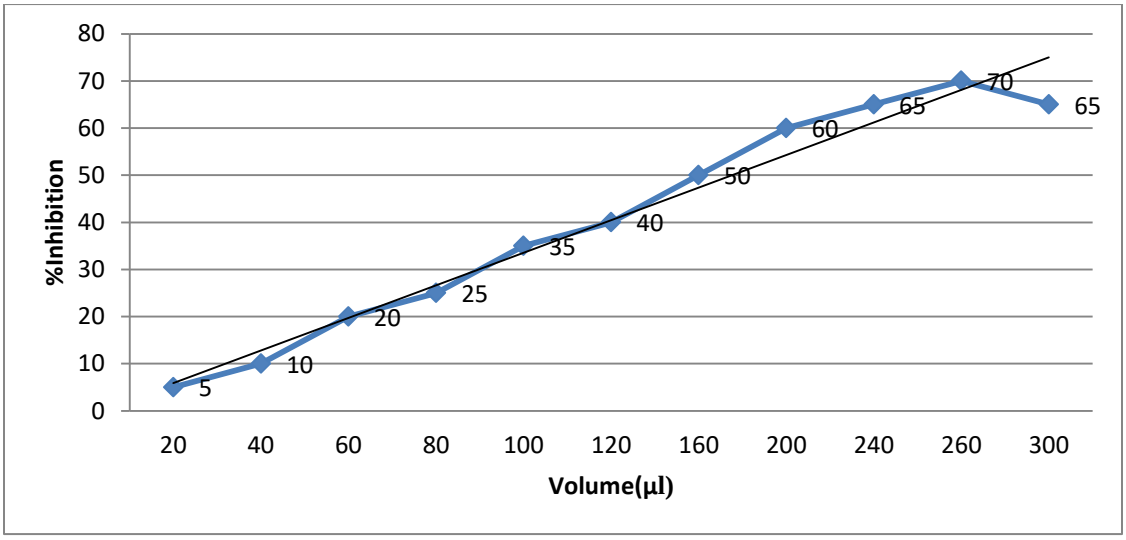


Fig. (2-11): Standard curve for the ratio of inhibition of the enzyme SOD

3. Calculate the half-inhibition ratio from the calibration curve.

1/2Inhibition=35%

4. The enzyme activity was calculated according to the following equation:
Activity of SOD enzyme(U/ml) =(inhibition% of sample)/(1/2 of the inhibition% from the standard curve 35%)

2.3.9. Determination of Potassium Concentration in Serum

2.3.9.1.Principle

Photometric Turbid metric test to determine Potassium ion concentration by using (Human Kit).Potassium ions in protein-free alkaline medium react with Sodium Teteaphenylboron to produce a finely dispersed turbid suspension of Potassium Tetraphenylboron. The turbidity produced is proportional to the Potassium concentration which can be measured photometrically at Wavelength (578nm) [126].

2.3.9.2 Regents Composition:

1	**Standard (Potassium K^+)**	**5 mmol/l**
2	**Trichloroacetic acid (TCA) Precipitant(Acid reagent)**	**0.3mol/l**
3	**Sodium tetraphenlboron (TPB-Na-reagent)**	**0.2mol/l**
4	**Sodium hydroxide NaOH (Alkaline reagent)**	**2 mol/l**

2.3.9.3. Deproteinization:

Pipette into centrifuge tubes (Potassium-free):

Reagent 2(TCA)	**1000 µl**
Serum	100 µl

It was mixed well and left to settle for 5 minutes and was placed in the centrifuge for 10 minutes, at 740xg /min ,a supernatant layer was used for measurement.

2.3.9.4. Procedure:

Working Reagent:

Prepared was added 1+1 mixture of reagent 3and 4 respectively in a volume enough for the assay stabled for 5 hours at $+15℃\ to\ +25℃$ when stored in the dark.

Reagents	Blank	Standard	Sample
Reagent 1	-	100 µl	-
Sample supernatant	-	-	100 µl
Working Reagent	1000 µl	1000 µl	1000 µl

Mixed well, it was left to rest for 5 minutes, at room temp. The absorbance of the sample and standard was read against the reagent blank at578nm.

2.3.9.5 Calculation:

Potassium Concentration:

$$\text{mmol/L} = \frac{Abs\ Sample}{Abs\ Standard} \times 5\ \text{mmol/l (con.Standard Solution)}$$

●Reference Values

Serum: 3.6-5.5 mmol/l

2.3.10. Determination of Calcium Concentration in Serum

2.3.10.1. Principle

Photometric test to determine calcium ion concentration using(Human Kit) which is mainly based on (CPC) Cresolphtalein complex. Calcium ions react with o-Cresolphthalein-complex in an alkaline medium to form a purple colored complex. The absorbance of this complex is proportional to the Calcium concentration in the sample [126].

Ca^{++} + CPC $\longrightarrow$ Ca-CPC complex

Complex absorbance measurement at a wavelength of 570nm.

2.3.10.2 Regents Composition:

1	100 ml Buffer Solution(BUF)
2	100ml Color Reagent(RGT)
3	3ml Standard (STD)

2.3.10.3. Reagent Preparation

To prepare the working solution, RGT and BUF were mixed in equal volumes. They were mixed and left to settle for 30 minutes at room temperature before use.

2.3.10.4. Procedure

1. Three sets of tubes (Sample, standard and blank) are prepared as the following:

	Blank	Standard	Sample
Working Reagent	1000 µl+20 D.W	1000 µl	1000 µl
Standard STD		20 µl	
Sample			20 µl

2. The solutions were mixed and incubated at least for 5 min at 37 ºC.

3.The absorbance of samples and standards are measured at wavelength 570 nm against blank.

2.3.10.5. Calculations

Concentration (mmoL/L) =Conc. St (2 mmoL/L)× $\frac{\Delta A\ sample}{\Delta AStandard}$

●Reference Values

Serum/ 2.2 - 2.6 mmoL/L

2.3.11 Determination of Sodium Concentration in Serum

2.3.11.1 Principle

Photometric Determination of Serum Sodium (Mg-Uranyl acetate Method) by using Human Kit, sodium was precipitated with Mg-uranyl acetate; the uranyl ions remaining in suspension form a yellow-brown complex with thioglycolic acid. The difference between reagent blank (without precipitation of sodium) and analysis had been proportional to the sodium concentration. Complex absorbance measurement at a wavelength of 410 nm [126].

2.3.11.2. Regents Composition:

1	**60 ml Precipitating solution (PREC)**	
	Uranyl acetate	**19 mmol/I**
	Magnesium acetate	**140mmol/l**
2	**60 ml Color reagent(RGT)**	
	Ammonium thioglycolate	**550 mmol/l**
	Ammonia	**550mmol/l**
3	**2 ml Standard Sodium(Na^+)**	**150mml/l**

2.3.11.3. Procedure

Reagent	Blank	Standard	Sample
Standard		20 µl	
Serum			20 µl
PREC		1000 µl	1000 µl

The Tubes were closed and mixed well and left to settle for 5 minutes, the tubes were shaken intensively for 30 sec, and left to settle for 30 min. The tubes were placed in the centrifuge at the highest speed for 5-10 minutes.

Reagent	Blank	Standard	Sample
PREC	20 µl		
Clear Supernatant		20 µl	20 µl
RGT	1000 µl	1000 µl	1000 µl

Mixed well, and after 5 minutes, the absorbance of the blank, standard and sample was measured against distilled water at a wavelength of 410 nm.

2.3.11.4. Calculations

Concentration = $150\times \frac{\Delta ARB-\Delta ASample}{\Delta ARB-\Delta ASTD}$ mmol/l

●Reference Values://135-155mmol/l

2.3.12. Statistical Analysis

The continuous variables were presented as mean and standard deviation (mean ±SD). T-test was used (Independent- sample T Test) to determine the mean differences between groups . The results were expressed as mean ±standards deviation for normally distributed values and 25-75% percentile (median) to express the results of nonparametric variables. Pearson's correlation coefficients were used to examine associations between scale variables and Spearman's correlation coefficient to find the correlation between nonparametric parameters and other variables.

Receiver operating characteristics (ROC) curves were measured to examine the diagnostic ability of the measured biomarkers to diagnose the disease . All statistical analyses were performed using IBM SPSS windows version 25, 2017. Figures and tables were plotted using the Excel software of Windows Office 2019.

Chapter Three

Result and Discussion

3. Results and Discussion

3.1. Comparison Study between T2DM Patients and Controls

3.1.1. Comparison of Demographic and Clinical Data

The baseline characteristics of the study groups in the Table (3-1) consist of the data of both the patients with DM and the control group; patients (n=60), (Female:22 & Male: 38), and Control (n=30) (Female:10 & Male:20). The mean ages of both groups were (44.47) and (47.55) for control and patient group respectively and no significant differences (P=0.397) were obtained.

Table (3-1): Demographic and clinical data of healthy controls and T2DM subjects

Parameters	Control	Patients	p-value
Age Yrs.	44.47 ± 8.760	47.55 ± 8.033	0.099
Height cm	170.63 ± 10.749	171.87 ± 9.146	0.571
Wt kg	75.53 ± 8.228	77.83 ± 7.293	0.180
BMI kg/m^2	25.960 ± 1.880	26.347 ± 1.441	0.281
Gender M/F	20/10	38/22	0.755
FBG mmol/L	5.593 ± 1.145	9.485 ± 2.201	<0.001
HbA1c %	5.135 ± 0 .718	7.639 ± 1.495	<0.001

Mean of the height in the control group was lower (170.63 cm) than in the patient group (171.8 cm) and also the mean weight in the control group was lower (75.53 kg) than patient group (77.83 kg), as well as mean BMI of the control group was lower (25.96 kg/m2) than patient group (26.34 kg/m2), and no significant differences were found.

Gender of participants was male to female (20 and 10) in the control group male to female in the patient group (38 and 22). It was found no significant change between two groups. A significant (p <0.001) was found in FBG in the

patient group when compared to control group was 5.593 mmol/L and 9.485 mmol/L respectively. High levels of fasting blood sugar among patient when compared to control suggest the body was not able to reduse blood sugar levels and this points to insulin resistance, and inadequate insulin production[127].

The amount of glycated hemoglobin (HbA1c) in the blood of a control group was estimated with that of a group of patients diabetes (type 2), and this the test gives the rate of increase in glucose levels for the past three months, and is considered a guide for control . The patient's blood sugar level and the results were as shown in Table (1-3) ,this means difficult regulation of glucose levels,and indicate diabetes Due to increasing binding hemoglobin to glucose in the blood stream and becomes glycated [128].

When comparing these results with other results, which is in line with results reported in Iraq by Fadhel and Yousif *(2019)* , found that glycated hemoglobin HbA1c, patients with type-2 diabetes had signifcantly higher compared to control group [129].

Gauda *et al.* concluded that HbAlc % better reflects the mean blood glucose concentration and degree of carbohydrate intolerance than fasting blood glucose levels and glucose tolerance tests, and may provide a better index of glycemic control in diabetic cases[130].

3.1.2. Comparison of Glutathione peroxidase between T2DM Patients and Controls

The results of GPx in healthy controls and T2DM patients are presented in Table (3-2) which showed the mean of GPx activity was (59.723) higher in the healthy control group than in the patient group was 46.556, These results showed significant differences ($p<0.001$) in GPX activity. Investigation found that T2DM participants had lower GPx activity.

Table(3-2): Comparison between T2DM patients and control in GPx

Parameter	Control	Patients	p-value
GPX U/mL	59.723 ± 17.249	46.556 ±11.812	<0.001

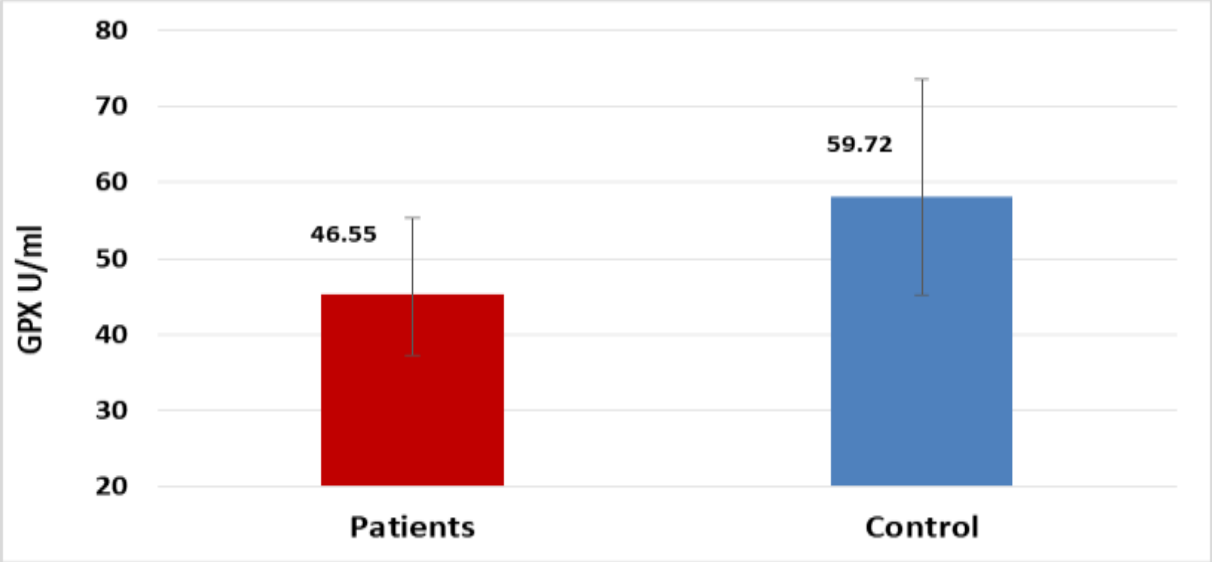


Fig.(3.1): Serum glutathione peroxidase in T2DM patients and control groups

Low activity of GPX for those who have T2DM related with excessive free radical generation that lead to decrease the activities of antioxidant enzymes and decrease in the GSH (substrate and a cofactor of GPX) and GPx. Thus, depletion of GSH impairs the activity of antioxidant enzymes as well as that of chain breaking aqueous and lipid phase antioxidants [131].

Reduced GPx activity is seen in conjunction with decreased SOD activity. Due to SOD have been reduced H_2O_2 generation, so GPx activity declines. However, elevated in superoxide radicals might inactivate GPx[132].

In comparison with other studies, this study full agree with a result reported in Iran that found the activity of GPX in patient DM T2 decreased without complication comparing to control [133]. while this study agree with result conducted by Leh et al., (2021) which found GPx of healthy group was 398.2 U/mL and 352.7U/mL for T2 DM group [134].

These findings recording lower GPx might be risk factor for type 2 diabetes and also the lower GPx activity may be because protein glycation, which is a mechanism that damages the protein of antioxidant enzymes.

3.1.3. Comparison of Superoxide Dismutase between T2DM Patients and Controls

The results of Antioxidant Superoxide Dismutase SOD in healthy controls and T2DM patients are presented in Table (3-3) . which showed that mean of SOD was (3.124U/L) in the healthy control group and in the patient with T2 DM group was (1.700U/L), The mean serum SOD level in the type 2 Diabetes mellitus was decreased as compared to the mean SOD activity in the control group, The difference between the both groups were statistically highly significant (P<0.001).

Table (3-3): Comparison between T2DM patients and control in SOD

Parameter	Control	Patients	p-value
SOD U/L	3.124 ± 0 .468	1.700 ± 0 .367	<0.001

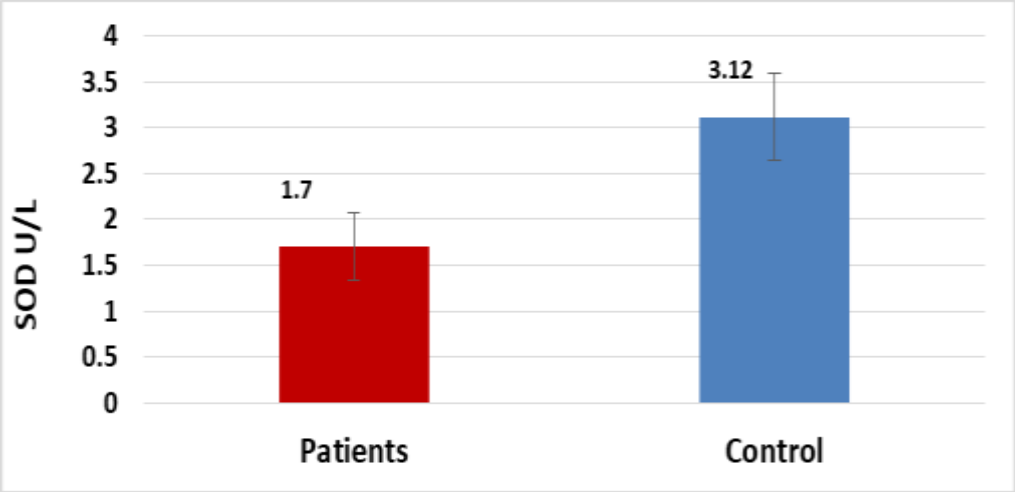


Fig.(3.2) Serum SOD in T2DM patients and control groups

The reasons of decrease of SOD activity in DM because hyperglycemia that activates biochemical pathways such as glucose auto oxidation, non-enzymatic glycosylation of proteins and activation of protein kinase C that led to produce oxidants like superoxide anion ($O_2^{\cdot-}$)and hydroxyl radicals and H_2O_2, as well as increase in glycosylated SOD that leads to inactivation of enzyme [135].

This result consistent with result reported by Basia et al., (2017) was found median levels of SOD in the healthy controls was 3.500 U/ml, while in patients of type 2 Diabetes mellitus without retinopathy group it was 1.500 U/ml with showed significant differences ($p<0.001$) in SOD activity [136] . As well agreed with many studies conducted in different countries that found decrease activity of SOD activates among DM patients [135].

3.1.4. Comparison of Malondialdehyde between T2DM Patients and Controls

The results of MDA in healthy controls and T2DM patients are presented in Table (3-4) and figure.(3.3) .which showed that mean of MDA was lower in the healthy control group (1.867) µMol/L than in the patient group (5.447) µMol/L, These results showed significant differences at ($p<0.001$) in MDA level.

Table (3-4):Comparison between T2DM patients and control in MDA

Parameter	Control	Patients	p-value
MDA µMol/L	1.867 ± 0 .465	5.447 ± 0 .790	<0.001

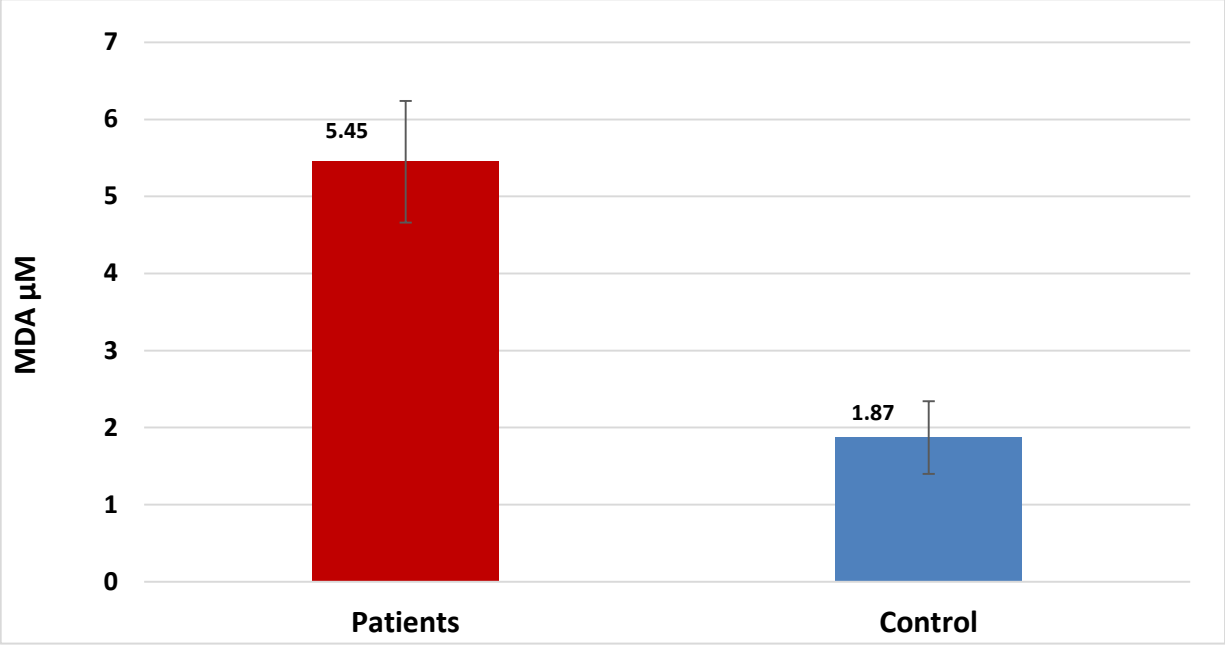


Fig.(3-3): Serum Malondialdehyde in T2DM patients and control groups

The most probable causes for the increased MDA levels in serum of diabetic groups may be due to the abnormal lipid metabolism. Hyperinsulinemia and hyperglycemia may increase free radical production and induce oxidative stress, which may contribute to an increased risk of MDA

serum levels in diabetes due to free oxygen radicals that cause peroxidative breakdown of phospholipids.

There are several studies supporting the theory of increased oxidative stress in diabetes mellitus by way of estimating MDA. These studies are agreement with the current study. A study conducted in Kurdistan/ Iraq that MDA level increased in diabetic group , when compared with control group [137], other study from Pondicherry have found a marked increase in MDA levels in diabetic patients with coronary heart disease in comparison to healthy controls [138] . A similar study in Mauritius has also reported significantly high malondialdehyde levels among a diabetic population [139], others reported a significant increase in MDA levels among Diabetic patients in comparison to the controls.The observed increase in malondialdehyde release might be attributed to the increase in peroxidative damage to lipids from oxidative stress developed during diabetes[140].

3.1.5. Comparison of Butyrylcholinesterase between T2DM Patients and Controls

The results of BuChE in healthy controls and T2DM patients are presented in Table(3-5) and figure(3-4),as (25-75%) percentile (median), which showed that mean of was lower in the healthy control group (13.127) than in the patient group (15.787), These results showed significant differences at ($p<0.001$) in BuChE level.

Table (3-5): Comparison between T2DM patients and control in BuChE

Parameter	Control	Patients	p-value
BuChE pg/ml	13.127(14.158-12.219)	15.787(14.278-17.657)	<0.001

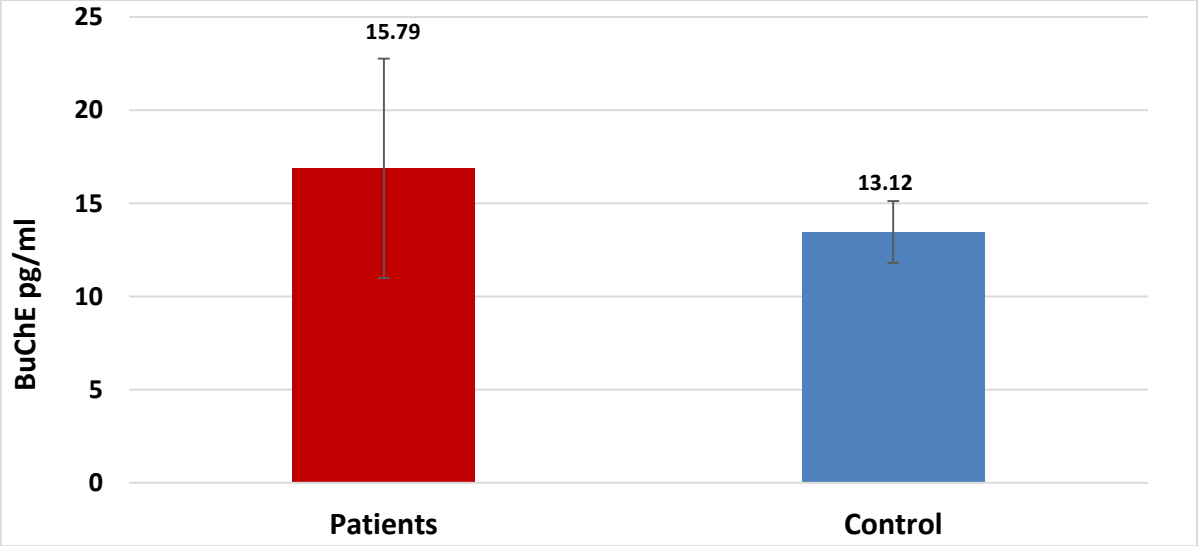


Fig.(3.4):Serum Butyrylcholinesterase in T2DM patients and control groups.

Serum butyrylcholinesterase levels are elevated could result in decreased acetylcholine levels and low-grade inflammation, which may play a role in the pathogenesis of type 2 diabetes linked to serum butyrylcholinesterase because insulin resistance is most likely caused by inflammation ,the explanation why BuChE high in T2 DM patients that Acetylcholine is act as anti-inflammatory that trigger the onset of systemic

inflammation in type 2 diabetes, lead to elevated of butyrylcholinesterase and acetylcholinesterase concentration[141].

Recently, suggested that increased in plasma and tissue activities of BuChE associated with different clinical conditions that make it a marker for systemic inflammation, as well as high level of plasma BuChE activity was significantly associated with type 1 and type 2 diabetes when compared to the control group. In addition, associated with serum of cholesterol, triglycerides and with overweight (obesity and body fat distribution)[142].

This results agreement with several studies, a cross sectional study conducted in China among adolescents reported high level of BuChE among obese and overweight subjects respectively when compared with under wight and normal weight respectively, as well as with high LDL-C and high BMI (BMI>25) among (Obese and Over wieght) that predicate to insulin resistance and lead to diabetes in adult age [143]. Other comparison study conducted among children with DM and without DM which found the level of BuChE activity among control subject was decrease and this level elevated in children with diabetes [144].

Reported by Tangvarasittichai et al.,(2015) that found BuChE level increas in control non obese women while BuChE level with high insulin level and low HDL was observed in obese women. Obesity results in insulin resistance, insulin dysregulation could act by decreased cortical glucose utilization, oxidative stress, formation of advanced glycated proteins, increased neurofibrillary formation and increased β-amyloid aggregation through inhibition of insulin-degrading enzyme. [145].

3.1.6. Comparison of Lipoic acid Synthetase and lipoic acid between T2DM Patients and Controls

The results of Lipoic acid Synthetase in healthy controls and T2DM patients are shown in the Table(3-6) Figure (3-5), (25-75%) percentile (median),which observed that the mean of Lipoic acid synthetase was higher in the healthy control group (11.660) than in the patient group with DM (8.774), and also observed that there is a significant association at ($p<0.001$) in Lipoic acid synthetase among subjects.

The results of Lipoic acid in healthy controls and T2DM patients are presented in Table(3-6) , Figure (3-6),as(25-75%)percentile (median) which showed that mean of Lipoic acid was lower in the healthy control group (0.380) than in the patient group (1.1839), there is no significant association at P- value.

Table 3.6 Comparison between T2DM patients and control in Lipoic acid and Lipoic acid Synthetase

Parameter	Control	Patients	p-value
Lipoic acid Synthetase pg/ml	11.660(13.387-10.190)	8.774(6.570-10.043)	<0.001
Lipoic acid	0.380±.01421	1.1839±1.12230	<0.052

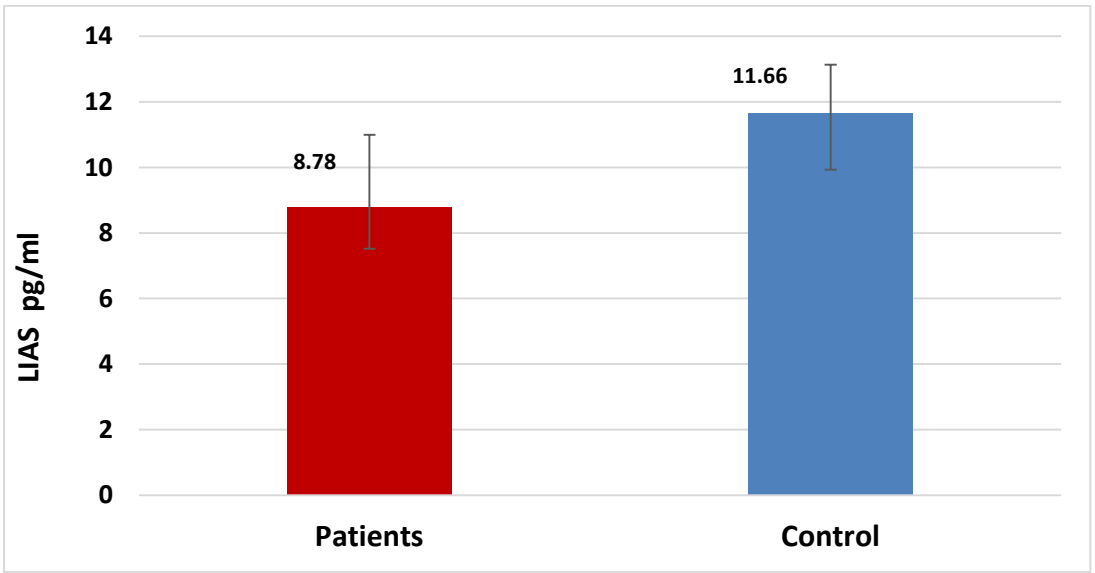


Fig.(3-5) :Serum lipoic acid synthetase in T2DM patients and control groups

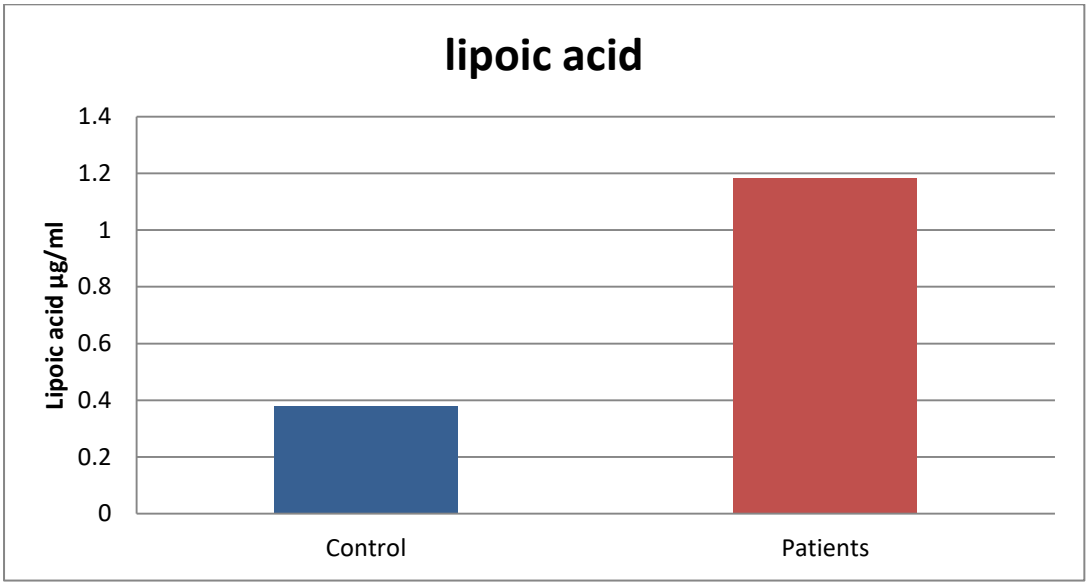


Fig.(3-6) :Serum lipoic acid in T2DM patients and control groups

Lipoic acid synthetase (LIAS) is the enzyme that is consider endogenous to synthesis of lipoic acid and also strengthen mitochondrial antioxidant, In mitochondria lipoic acid synthase produces α-lipoic acid, antioxidant and cofactor in a ketoacid dehydrogenase complexes which participate in glucose oxidation [146].

Excessive levels of ROS, such as superoxide anion, can cause direct damage to cellular macromolecules. Because mitochondria are a major source of ROS, accumulated ROS could damage the mitochondrial membrane. This may then result in mitochondrial dysfunction. The decrease in mitochondrial membrane potential observed in our in *vitro* LIAS knockdown studies could be the result of ROS damage.Increased circulating inflammatory cytokines and mitochondrial dysfunction both contribute to insulin resistance[147].

The elevated in lipoic acid among DM patient that because decrease in LIAS enzyme that responsible to synthesis of lipoic acid led to increase of

lipoic acid among DM patient and also they may rely on regimen diet or use specific medication lead to increase lipoic acid in serum. Alpha lipoic acid is effective in many pathological conditions where ROS have been implicated, including diabetes mellitus. LA is synthesized in the liver and other tissues, and it is obtained in the diet from both animal and plant sources.LA is rapidly absorbed from the gastrointestinal tract and significantly raises plasma LA levels[148].

Deficiency of LA synthetase can be caused either by mutations in the structural gene LIAS or by deficiency in the synthesis of the [4Fe-4S] FeS cluster cofactors. Defects in LA synthetase result in a decrease in lipoylation of all lipoyl-containing enzymes, including the 2-oxoacid dehydrogenases and the glycine cleavage system GCS [85].

Increased oxidative stress is a feature of diabetes mellitus, and it has a deleterious impact on mitochondrial integrity and function. As a result of its participation in lipoic acid production, LIAS has physiologic significance in diabetes [149].

Several studies conducted experimental studies also involved laboratory animals and concluded that the lower lipoic acid synthetase predicted to diabetes mellites and associated with diabetes nephropathy [146], [148].

The reason of the level of lipoic acid increased in the patients with DMT2 it’s may due to liberated of lipoic acid molecule from destroyed the phospholipid cell membrane after the effect of increasing the oxidative stress in the case of T2 DM.

3.1.7. Comparison of β-catenin between T2DM Patients and Controls

β-catenin in the present study among healthy controls and T2DM patients are shown in the Table(3.7) , figure (3.7),as (25-75%)percentile (median). the current study revealed that the mean of β-catenin had no obvious difference between the healthy control group (5.622 ng/ml) and the patient group (5.130 ng/ml) with significant association at ($p<0.01$) in β-catenin level among participants.

Table(3-7): Comparison between T2DM patients and control in β-catenin

Parameter	Control	Patients	p-value
β-catenin ng/ml	5.622(10.151-3.554)	5.130(4.169-9.828)	<0.01

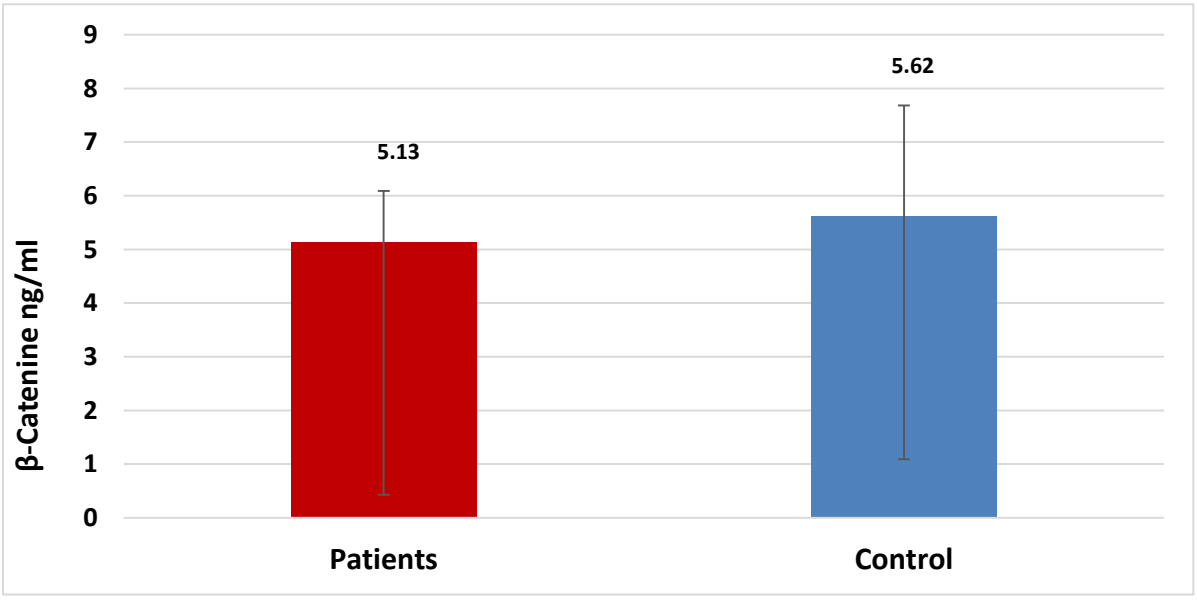


Figure (3-7) Serum β-catenine in T2DM patients and control groups

Many experimental studies attempt to prove association between β-catenin and DM, experimental study conducted by Dorfman *et al.* , (2015) among laboratory animals (rats), and results of this study are disagree with the

results of our study which found diabetic rats showed a significant increase in β-catenin protein compared with control group and enhanced intestinal epithelial cell proliferation in diabetic rats correlates with β-catenin accumulation [150].

In comparison with other studies, this result of β-catenin among DM participant was higher than result reported by Gaudio *et al*., (2012) that found of β-catenin among DM patient was (2.43 ng/ml), and this result agreed with same study regarding control group which was (5.94 ng/ml) and this differences in result belong to differences in target population that study conducted by Gaudio et al., (2012) targeted older age group and most of them sedentary with duration of DM 10 years or more that clear varies between β-catenin [151].

Experimental reported by Stahl, (2017) concluded that canonical abnormalities in Wnt/β-catenin pathway associated with cancer and diabetes. Ones of the abnormalities lead to increase Wnt signaling resulting in increased ß-catenin within the cell. Abnormal glucose levels is characteristic of diabetic patients and proposed to trigger the up-regulation of the Wnt/ ß-catenin pathway [152]. As well as another trail showed that Wnt/βcatenin signaling pathway as prominent a one of the most signaling pathway regulated by increased glucose in media and there was relationship between hyperglycemia and gene expression of the Wnt/ ß-catenin signaling pathway [153].

H. Chen *et al*., (2021) proved hyperglycemia exacerbated the abnormal activation of β-catenin in Pancreatic ductal adenocarcinoma (PDAC) and hyperglycemia accelerated the formation of precancerous pancreatic lesions by activating the Wnt /β-catenin signaling pathway in *vivo* and in *vitro* [154].

All previous studies proved any deterioration in β-catenin may generate disorder in glycemic that explain disparity in results, substantial evidence indicates that β-catenin is a pivotal regulator that contributes to the initiation

and development of various types of diseases. Recently, β-catenin can be detected in human serum and also reported to be correlated with several disease progression with little research regarding diabetic and most of them is still experimental.

3.1.8. Comparison of electrolytes between T2DM Patients and Controls

Electrolytes play an important role in several body mechanisms, to name a few it helps maintain acid base balance, membrane potential, muscle contraction, nerve conduction and control body fluid. Alterations in electrolytes homeostasis may lead to physiologic disorders [155].

The results of electrolytes in healthy controls and T2DM patients are presented in Table (3-8).

Table(3-8): Serum electrolytes levels of Diabetes subjects and healthy controls

Biomarker	Control	Patients	p-value
Ca mM	**2.313 ± 0.273**	**1.976 ± 0.302**	**<0.001**
K mM	**4.780 ± 0.435**	**5.101 ± 0.563**	**0.007**
Na mM	**142.226 ± 11.487**	**144.692 ± 13.318**	**0.389**

Measurement of the mean Calcium (Ca ion) in the healthy control group was 2.313 mM while in T2 DM patients was 1.976 mM and there was a significant association at P- value < 0.001, as well as the mean potassium in the healthy group was 4.780 mM and elevated to (5.101) in T2 DM patients and there is no statistical association, regarding sodium mean of (Na ion) was 142.226 mM in the healthy group and (144.692mM) in the patient group also there was no statistical association.

These differences in electrolytes measures among patient and control groups according to several studies related to Insulin activate Na^+ /K^+ -ATPase enzyme so that the low serum insulin lead to reduce Na^+ /K^+ -ATPase activity with poor Na^+ and K^+ metabolism[155].

Differences in insulin levels cause Ca+2 oscillations even in Ca+2-free medium; insulin increases cytoplasmic Ca+2 by mobilizing intracellular Ca+2 stores.

The results of our study are agree with the results of study finding by Liamis *et al*., (2014) a case control study conducted in Kanchipuram (India), Liamis *et al*.,(2014) found the level of(Na, K) ions was decrease in DM pateints compare with healthy groups and no sgnificant asociation [156]. And these results disagree with results conducted by Unachukwu *et al*., (2018) in india, who found elevated level of Na ion in patient group with DM type II to 153.6 mM [157]. study by Yasmin F *et al*, they found lower levels of all electrolytes *i.e*. sodium, potassium, chloride, calcium, magnesium and phosphorus in patient with DM [158]. Since majority of these studies were done in countries having different race and genetics and according or these differences in Electrolytes may be due to hyperglycaemia, ketoacidosis or renal dysfunction or even administration of drugs such as diuretics, like Amiloride .

3.2. Comparison of All Data between Good (HbA1c<7) and Poor (HbA1c>7) Glycemic T2DM Patients

Table (3-9) demonstrated comparison between type2 diabetes mullites patients, the mean age of good and poor control was (47.23 and 47.79) and BMI was (26.507 and 26.225) respectively, with no statistically significant (P>0.001).

Table (3.9): Comparison of all data between good (HbA1c<7) and poor (HbA1c>7) glycemic T2DM patients

Parameters	Good Control (HbA1c<7)	Poor Control (HbA1c>7)	p
Age Yrs.	47.23 ± 7.350	47.79 ±8.619	0.790
Height cm	171.04 ± 9.053	172.50 ± 9.301	0.544
Wt kg	77.58 ± 7.543	78.03 ± 7.205	0.814
BMI kg/m^2	26.507 ± 1.427	26.225 ± 1.460	0.459
Sex M/F	42.27 ± 6.453	41.44 ± 6.561	0.627
Ca mM	2.050 ± 0.308	1.880 ± 0.271	0.030
K mM	5.029 ± 0.683	5.157 ± 0.453	0.387
Na mM	146.950 ±11.486	142.965 ± 14.494	0.254
GPX U/Ml	44.144 ±10.446	48.401 ±12.597	0.169
MDA µmol/l	5.616 ± 0.720	5.317 ± 0.826	0.147
SOD u/ml	1.684 ± 0.397	1.713 ± 0.348	0.765
FBG mM	7.450 ± 0.709	11.041 ± 1.589	<0.001
HbA1c %	6.299 ± 0.444	8.663 ± 1.163	<0.001
Duration yrs.	4.96 ± 2.835	6.35 ± 4.277	0.157
Lipoic acid pg/ml	10.412(8.800-12.415)	8.648(6.713-9.413)	0.332
BuChE pg/ml	14.032(12.924-16.295)	15.515(16.566-14.279)	0.395
β-catenin ng/ml	5.655(4.077-10.304)	4.913(3.815-8.650)	0.161

There was no significant association (P> 0.001) was demonstrated of serum electrolytes (Ca, K, Na) with antioxidative (GPX, SOD) and MDA for good and poor HbA1c patients. While there was significant association (P<0.001) between FBG and HbA1c for good and bad HbA1c and means of FBG and HbA1c were (7.450, 11.041) and (6.299, 8.663) for good and poor control of HbA1c respectively.

In comparison with other studies, this study agreed with Khader, *et al*., (2018) that reported Anthropometric profile according to BMI (16.1%) had good glycemic control and (83.9%) had poor glycemic control and It was found that in age group below 50 years (11.1%) had good glycemic control and(88.8%) had poor glycemic control, In age group 50 to 59 years (8.8%) had good glycemic control while above 60 years (4.2%) patients had good glycemic control [159], unlike finding conducted by Radwan *et al*., 2018 found older age was associated with good glycemic control [160].

A higher prevalence of poor glycemic control was present among people with more than five years' duration of diabetes ($p = 0.005$) and no significant association regarding BMI of under, normal, over weight and obese for good and bad HbA1c [161].

This study consistence with study conducted by Abera *et al*., (2022); reported that patients without established glycemic target goals were found to be (3.42) times more likely to have poor glycemic control than those who established one with significant association ($p>0.001$) [162]. Fekadu *et al*., (2019) showed that older age of individuals with diabetes have poor control their blood sugar compared to those younger [163]. As well as that Anioke *et al*., (2019) reported majority (83.3%) had poor glycemic control status of which about (95%) constitute the elderly. The elderly was more likely associated with poor glycemic control than the non-elderly ($p = 0.006$). Age and Fasting plasma glucose (FPG) although attenuated the odds (odd ratio= 5.00, 95% CI: 1.19-20.96) of poor glycemic control [164].

Gebrie *et al*., (2022) and Abera *et al*., (2022) which found patients with a longer duration of DM more likely to have poor glycemic control than those with a shorter duration of the disease. That might be due to the chronic disease and progressive of diabetes so that patients with a longer duration of DM may

difficult maintain their glycemic as well Impaired insulin because of beta-cell dysfunction [165],[155].

Tekalegn *et al*., (2018) and Alemayehu *et al*., (2020) found that patients who did not set target goals for glycemic management more likely to have poor glycemic control than those with an established plan. This may be a lack of awareness of target blood glucose levels to manage diabetes among diabetes patients [166],[167] .

This findings disagreed with findings conducted by Palekar & Ray, (2016) was found that MDA be significantly lower in patients with good (HbA1c <7.0%) as compared to those with poor HbA1c, while agreed with same study that found superoxide dismutase (SOD) and glutathione peroxidase (GPX) levels were lower in patients with good glycemic control as compared to patients with poor HbA1c >7.0% [168] .

3.3. Correlation Study

3.3.1. Correlation Among the Parameter's Levels of Subjects

The Table (3.10) demonstrated the correlation between biomarkers which showed strong positive correlation between GPX with LIAS and Ca ion and weak negative correlation with MDA (0.304, 0.273 and – 0.239) respectively, as shown in the figures (3-8,3-9), LIAS had positive correlation with SOD and Ca ion . Weak positive with lipoic acid while negative correlation between LIAS with BuChE ,MDA and k ion (0.529, 0.219,0.898, -0.230 -0.425 and -0.296) respectively as shown in figures (3-10,3-111,3-12).

Current study showed positive correlation between BuChE with MDA and K ion and negative correlation between BuChE and SOD (0.465, 0.325, -

0.445 respectively) as shown in figures (3-13,3-14,3-15). while no correlation found between beta catenin and other Parameters.

Beside beta catenin, this study elucidated negative and positive correlation between MDA with SOD, Ca (-0.671, -0.430) and positive correlation between MDA with K ion (0.283) , that demonstrated in figures (3-16,3-17,3-18) as well as there was strong positive correlation between SOD and Ca ion (0.446) and negative between K with Na ions (0.750) demonstrated in figures (3-19,3-20).

In comparison with other studies, this study inconsistent with study conducted by PERSIKE, (2020) there was no correlation between BuChE and MDA [169], while J. Chen *et al*., (2021) reported a strong correlation between Wnt/ beta catenin signaling pathways and T2DM [170], Omu *et al*., (2010) reported that Serum and placental butyrylcholinesterase activity (BuChE) showed a strong inverse correlation with malondialdehyde (MDA), this results disagree with present study that found positive correlation between (BuChE and MDA), Omu *et al*., (2010) reported strong positive correlation between BuChE with total antioxidant activity in serum showed that Butyrylcholinesterase s2reducing oxidative stress in diabetic pregnancy[171] .Verma *et al.*, (2016) demonstrated in his study that there was no association between SOD and MDA (0.001) (0.704) in DM group and control group [172].

The current study concurred with the study conducted by SAITO *et al*., (1999) that reported inverse relationship between Na and K ions levels in DM patients [173].

This differences in results that DM type 2 based on several sociodemographic characteristic of individual like age, weight, sex, physical activities, life style and even genetics that clear association with chronic disease as well as DM effected by economic status and education level of patients , and

there was no previous studies that studied association between beta- catenin and BuChE, while fewer studies that studied correlation between oxidative stress and the correlation between serum electrolytes.

Table(3-10): Correlation among the Parameter's levels of subjects

Parameter	GPX	LIAS	BuChE	B-catenin	MDA	SOD	Ca ion	K ion	Na ion	Lipoic acid
GPX	1	0.304**	-0.037	0.027	-0.239*	0.153	0.273**	-0.022	-0.136	-.115
LIAS	0.304**	1	-0.230*	0.106	-0.425**	0.529**	0.219*	-0.296**	0.008	.898*
BuChE	-0.037	-0.230*	1	0.082	0.465**	-0.445**	-0.199	0.325**	-0.104	-.005
β-catenin	0.027	0.106	0.082	1	0.05	0.013	-0.132	-0.054	0.022	-.012
MDA	-0.239*	-0.425**	0.465**	0.05	1	-0.671**	-0.430**	0.283**	0.028	-.465
SOD	0.153	0.529**	-0.445**	0.013	-0.671**	1	0.466*	-0.203	-0.141	0.062
Ca ion	0.273**	0.219*	-0.199	-0.132	-0.430**	0.466**	1	-0.171	-0.075	0.210
K ion	-0.022	-0.296**	0.325**	-0.054	0.283**	-0.203	-0.171	1	0.750**	-0.145
Na ion	-0.136	0.008	-0.104	0.022	0.028	-0.075	-0.075	0.750**	1	0.284
Lipoic acid	-.115	.898*	-.005	-.012	-.465	0.062	0.210	-0.145	0.284	1

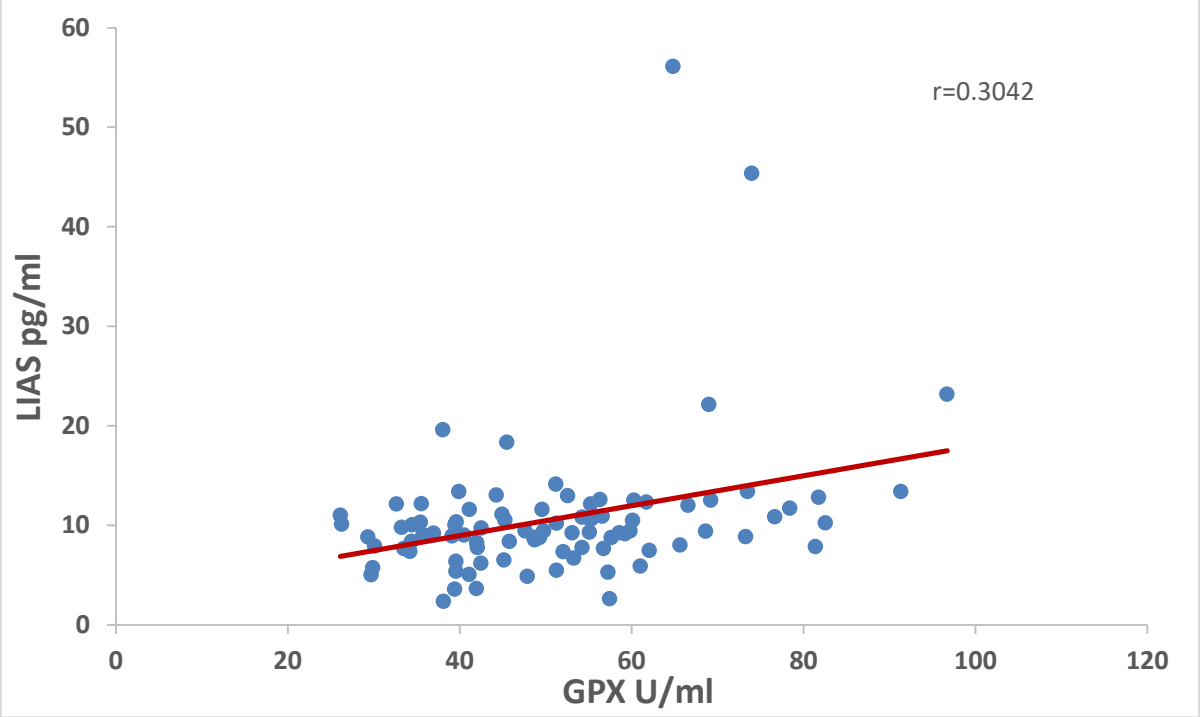


Fig.(3-8): Correlation between serum level of lipoic acid synthetase and glutathione peroxidase

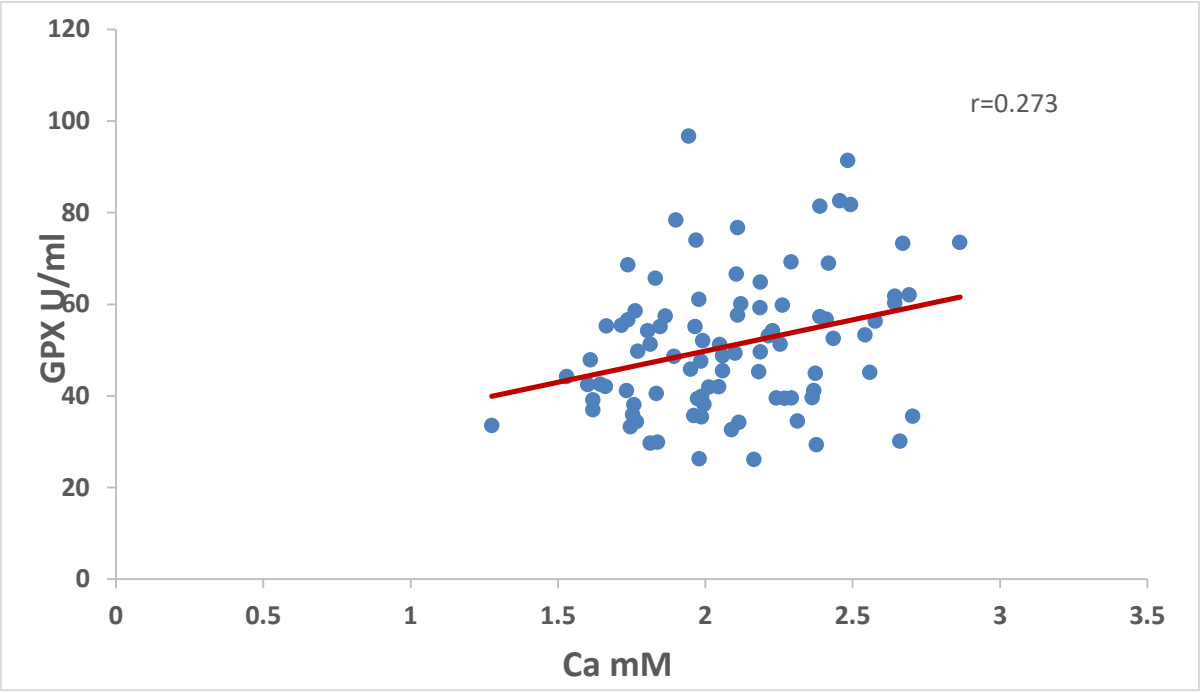


Fig.(3-9): Correlation between serum level of glutathione peroxidase and Calcium

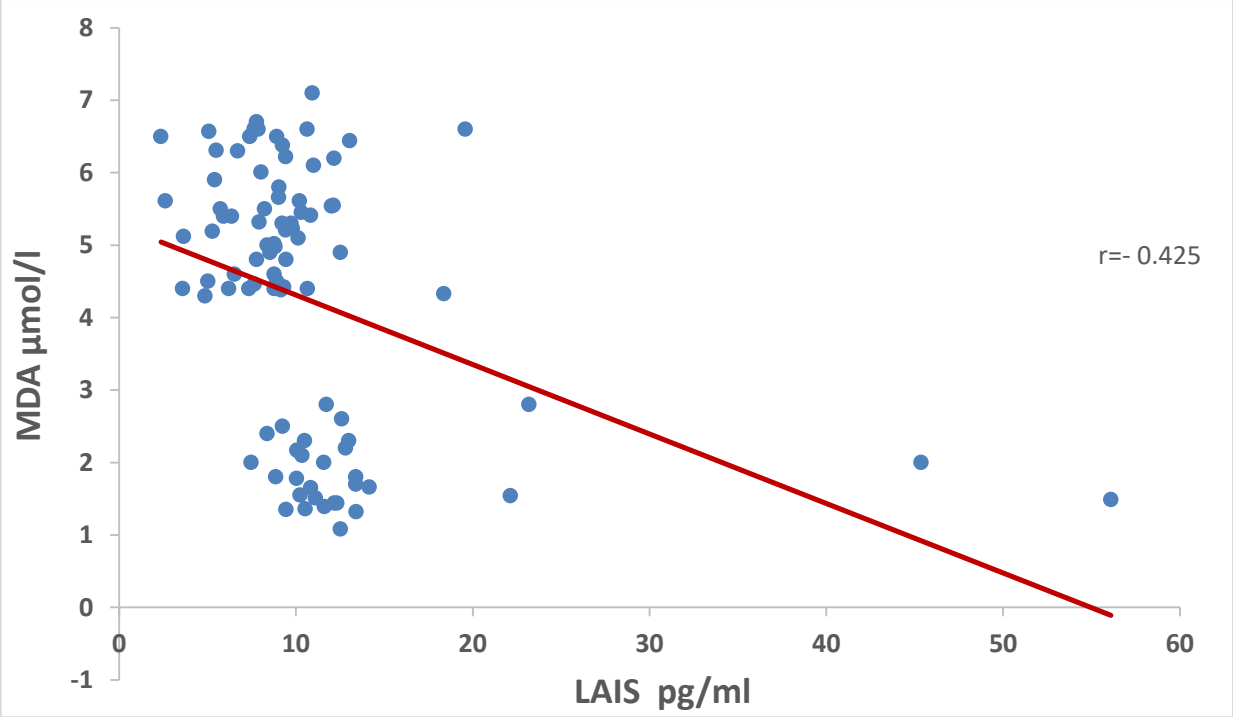


Fig. (3-10): Correlation between serum level of malondialdehyde and lipoic acid synthetase

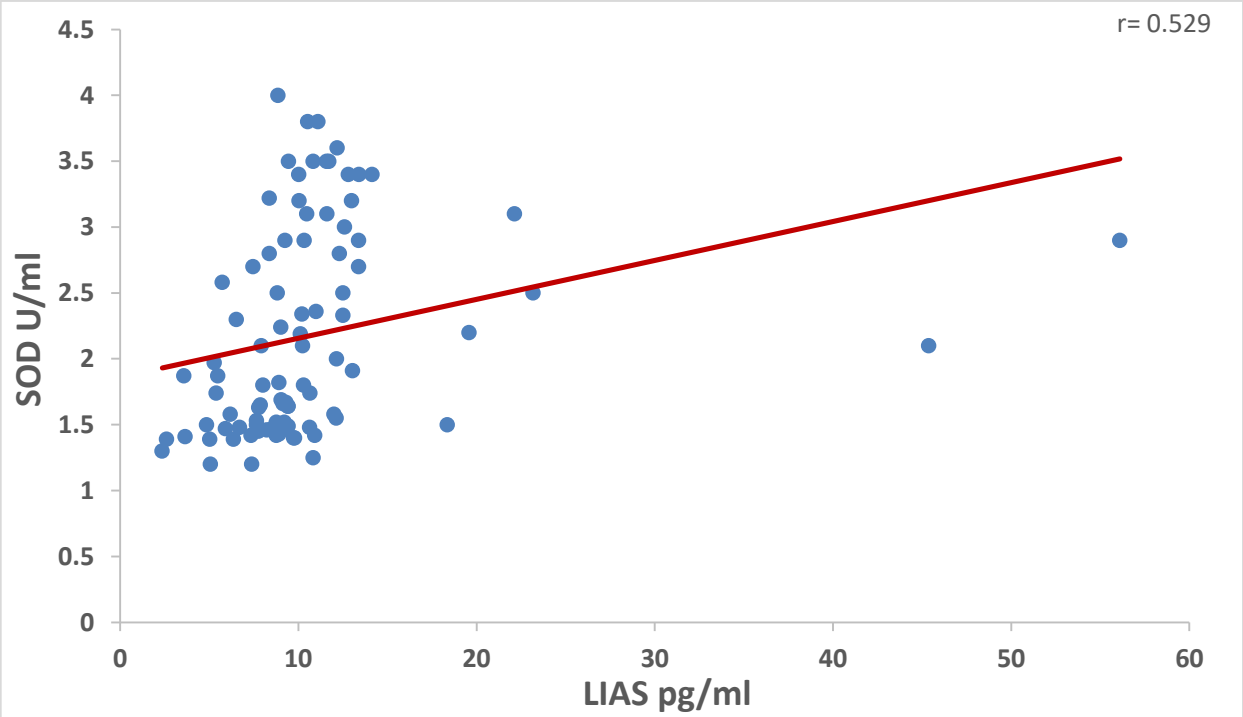


Fig.(3-11): Correlation between serum level of lipoic acid synthetase and Superoxide dismutase

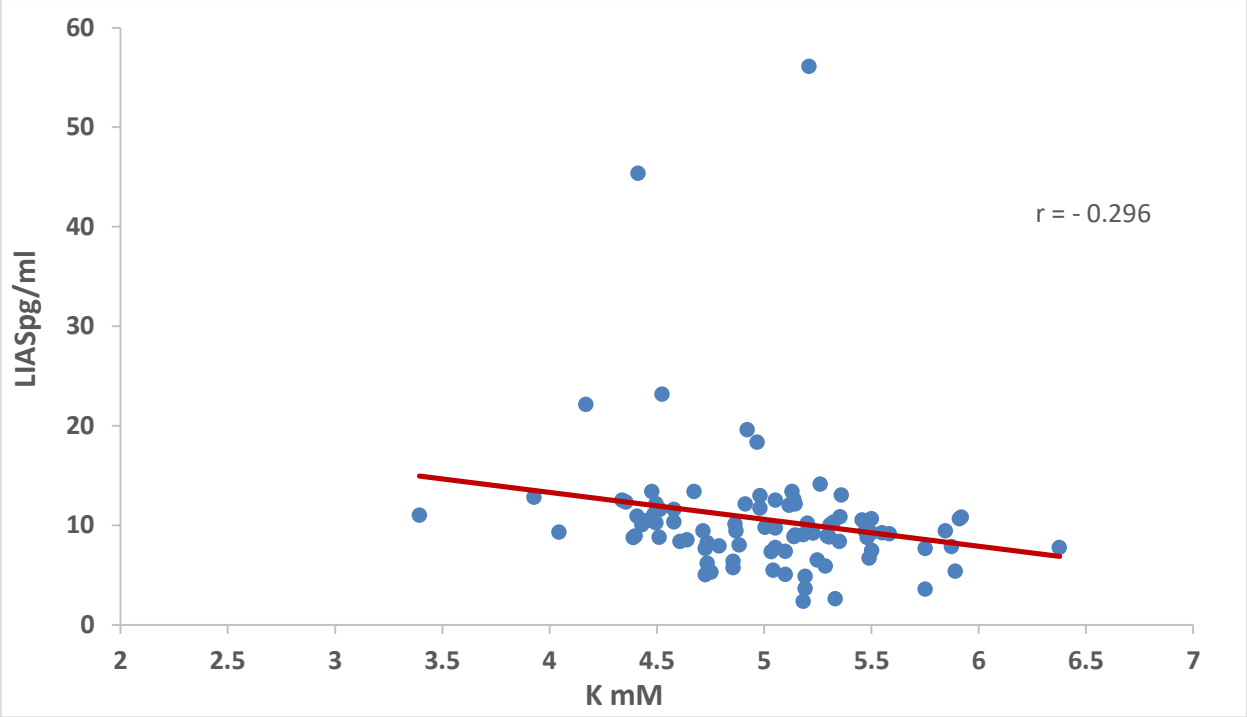


Fig.(3-12): Correlation between serum level of lipoic acid synthetase and potassium.

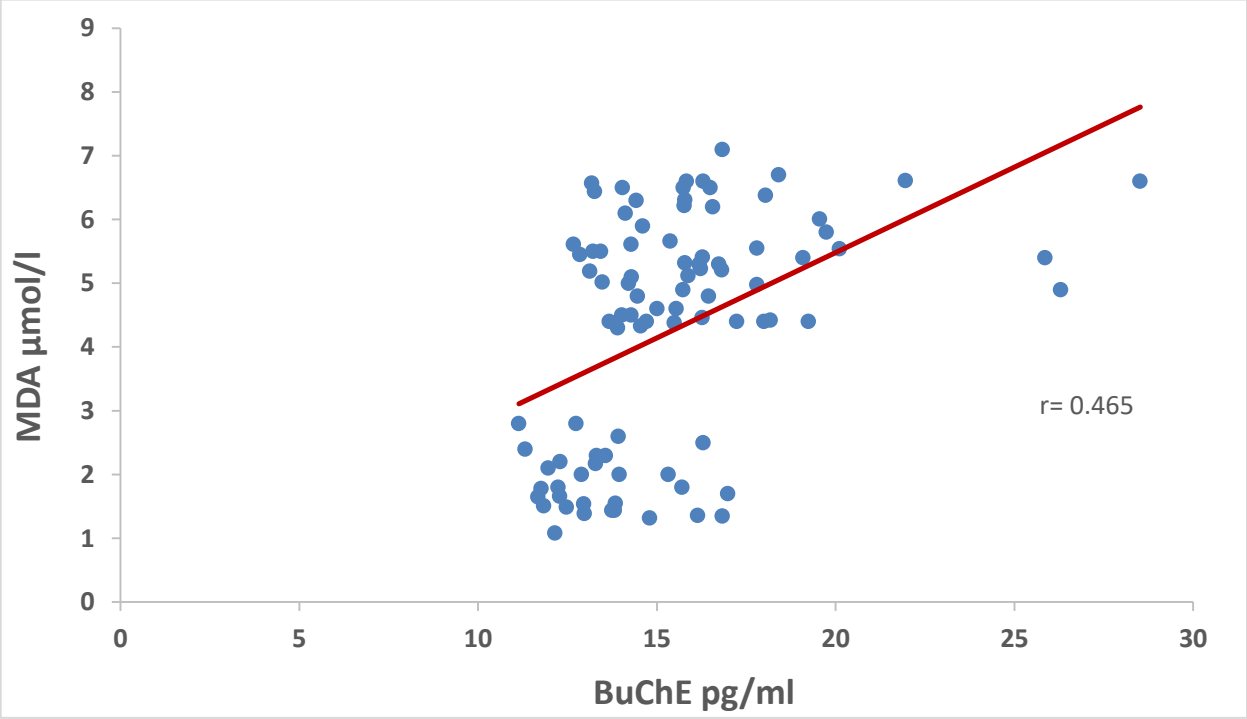


Fig.(3-13): Correlation between serum level of malondialdehyde and BuChE

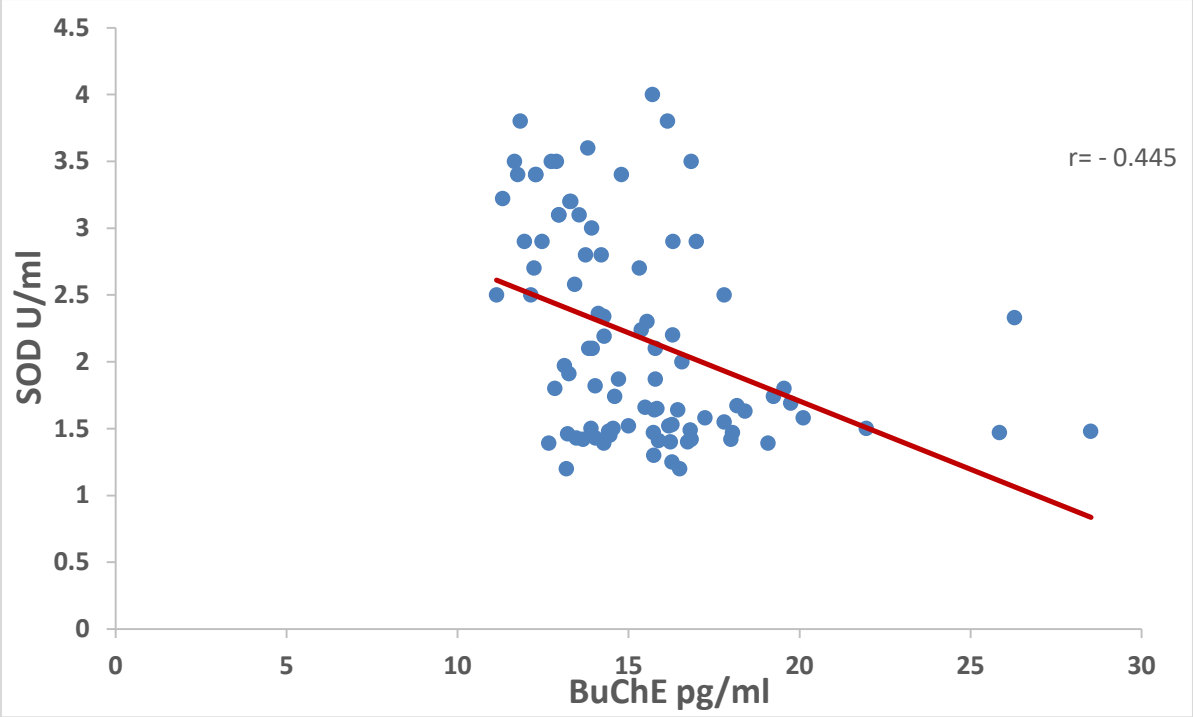


Fig.(3-14): Correlation between serum level of Superoxide dismutase and BuChE

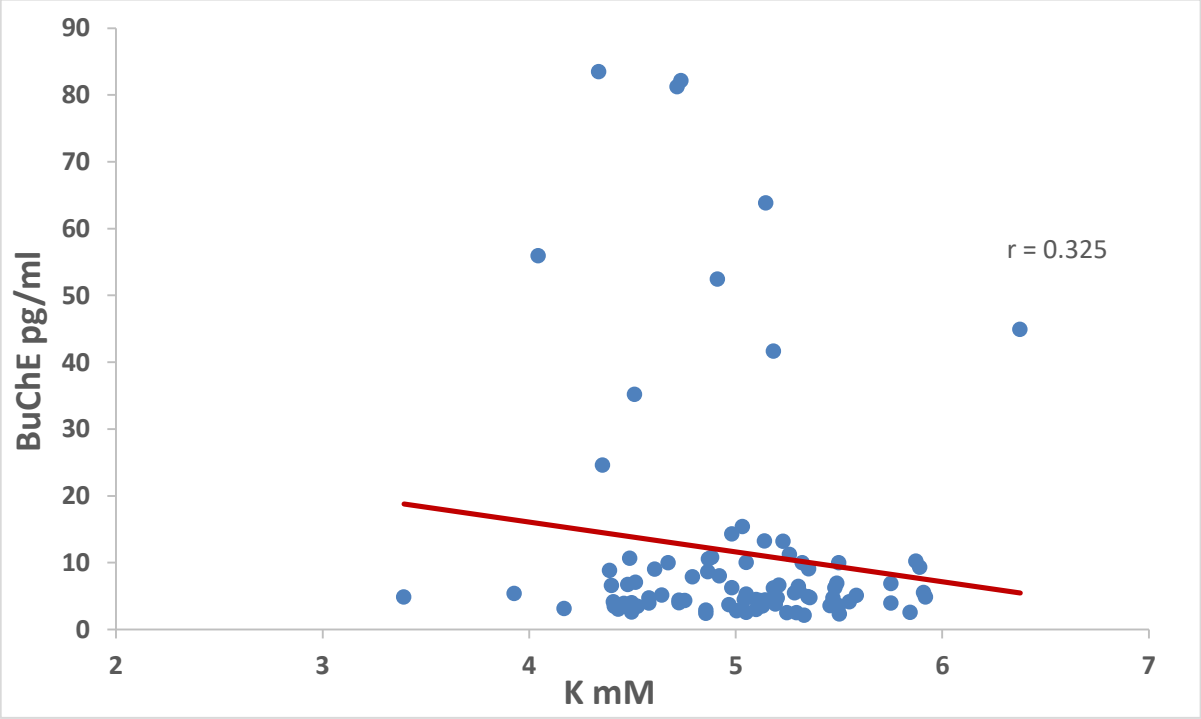


Fig.(3-15): Correlation between serum level of BuChE and potassium

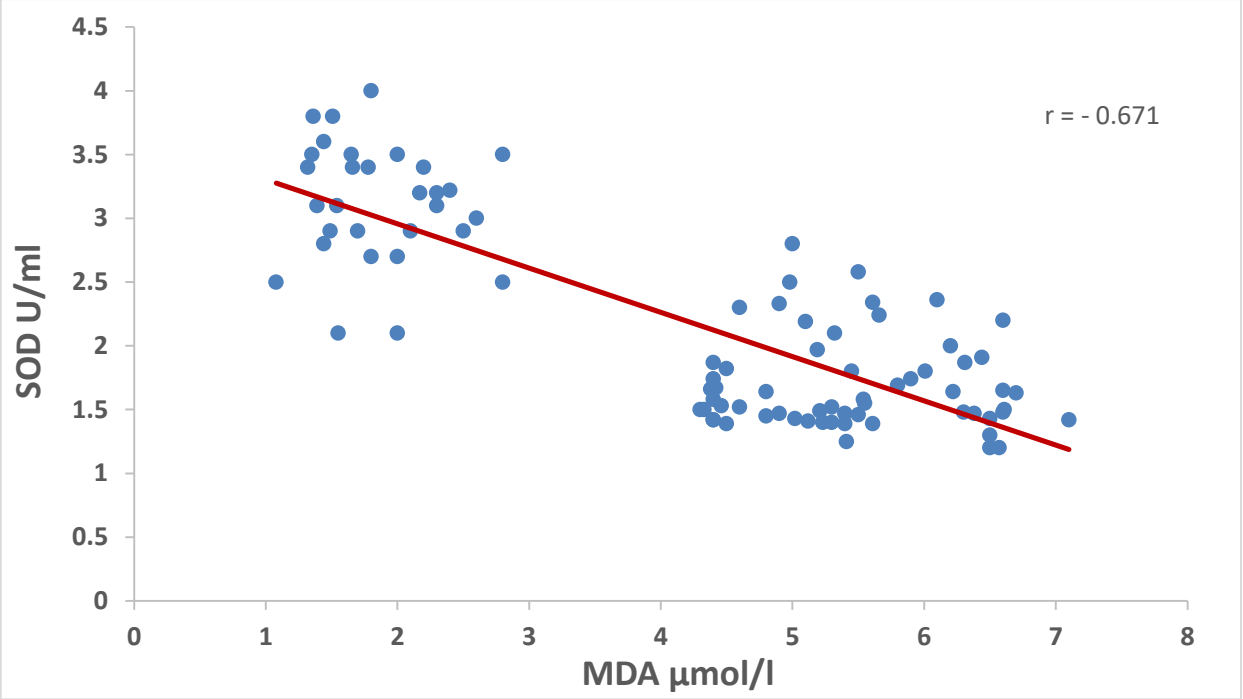


Fig. (3-16): Correlation between serum level of Superoxide dismutase and malondialdehyde

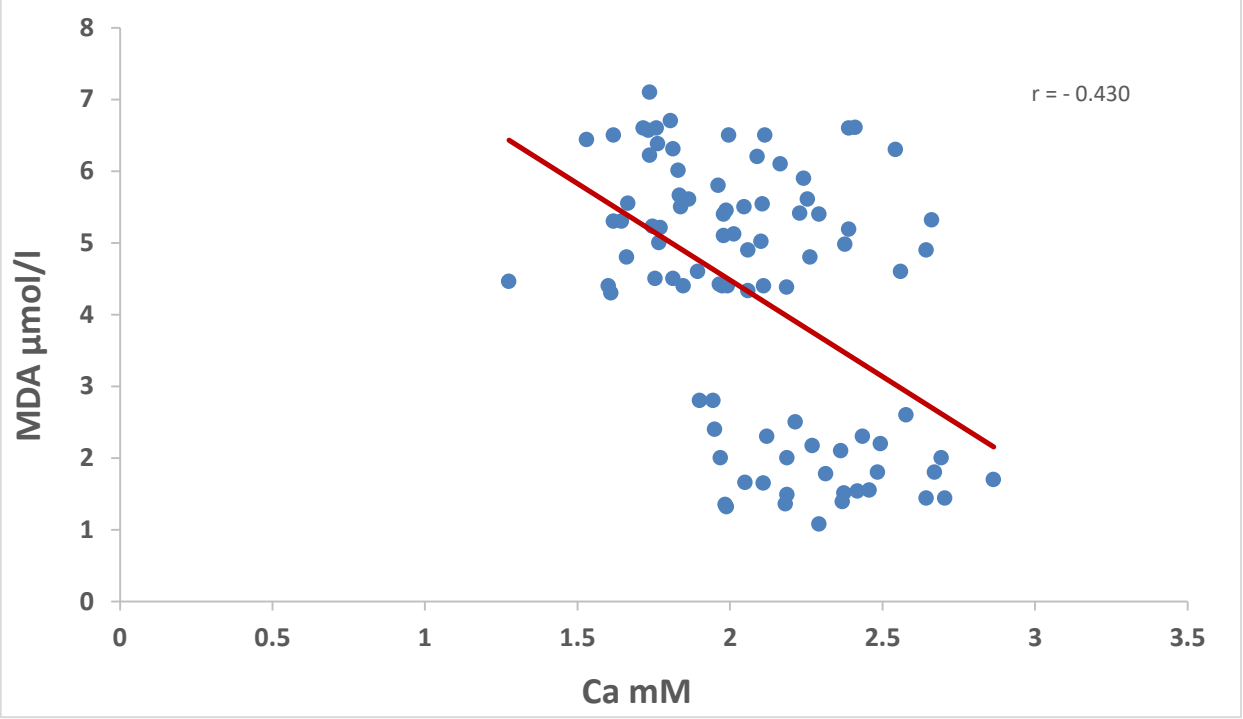


Fig. (3-17): Correlation between serum level of malondialdehyde and Calcium

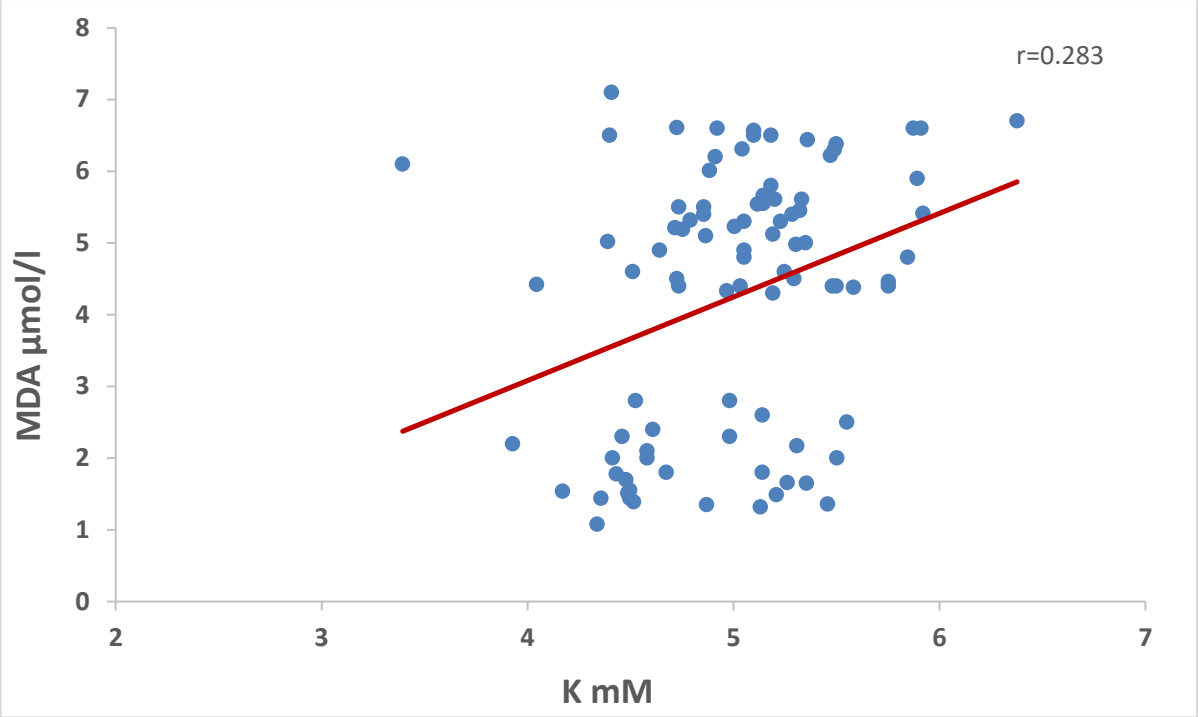


Fig.(3-18): Correlation between serum level of Malondialdehyde and potassium

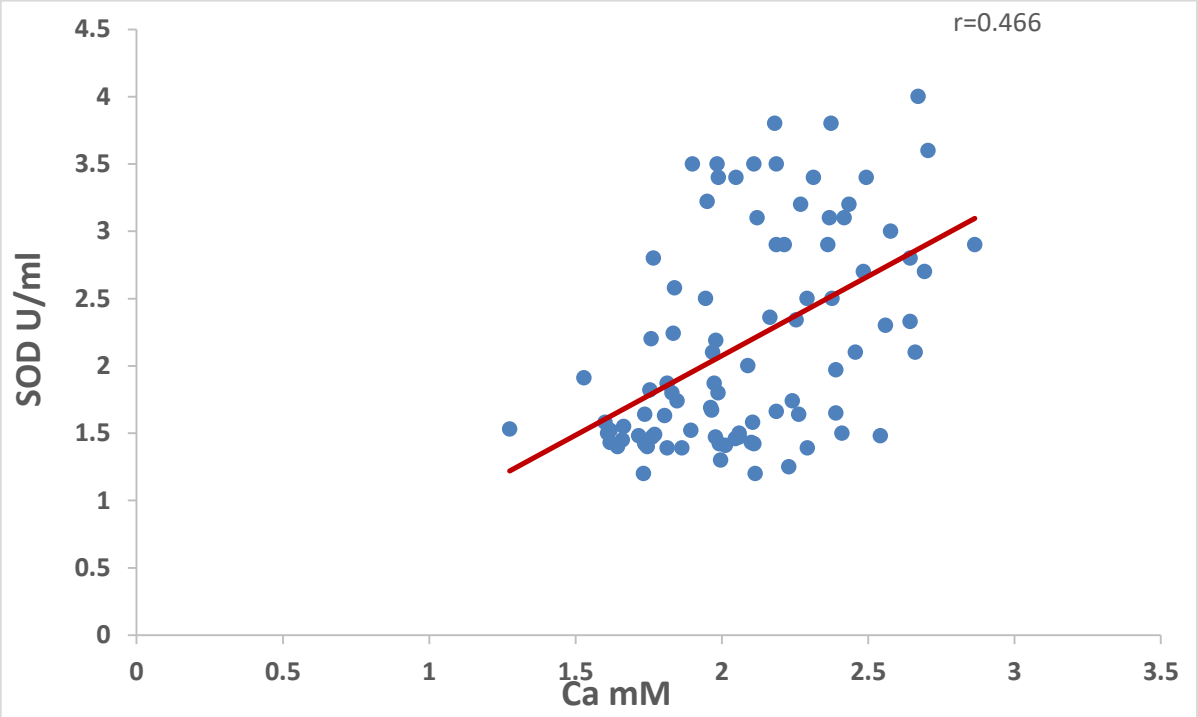


Fig. (3-19): Correlation between serum level of Superoxide dismutase and Calcium

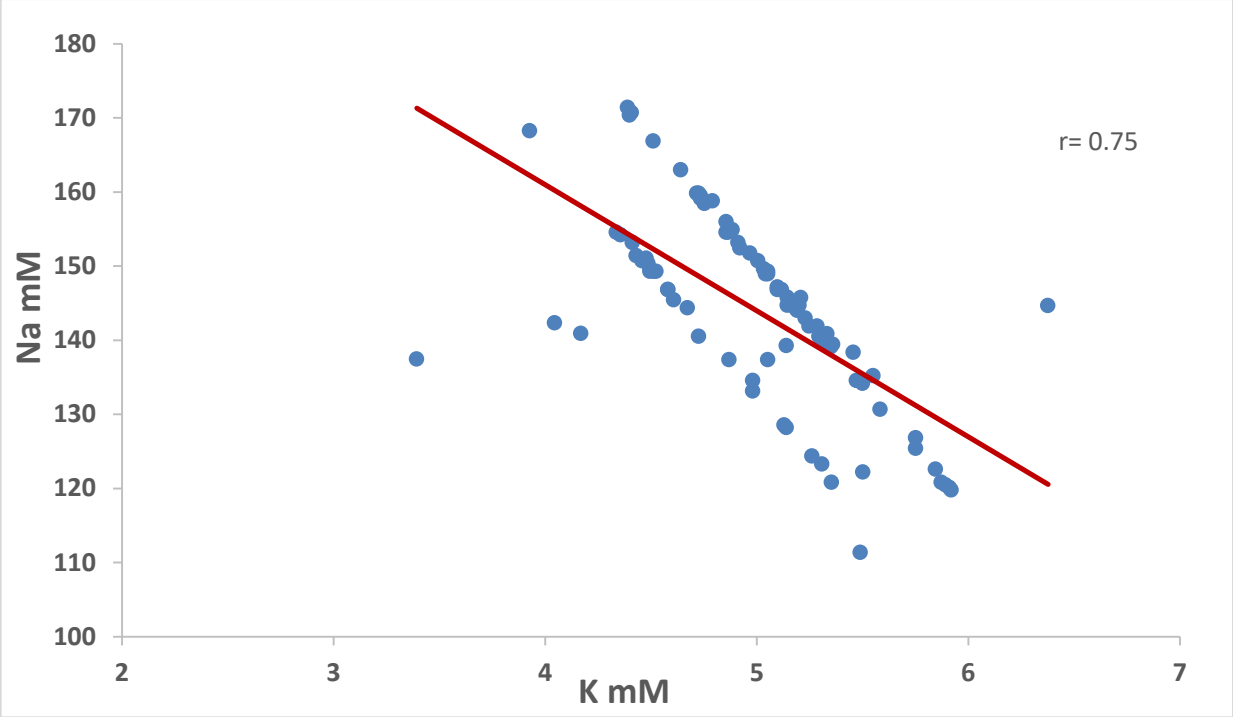


Fig.(3-20): Correlation between serum level of sodium and potassium

3.3.2. Correlation between the Parameters and the electrolytes of subjects.

In the current study, there was negative correlation between FBG with Ca, Na ion, GPX, lipoic acid and SOD (-0.260, -0.039, -0.269, -0.419 and -0.531) respectively, and positive correlation between FBG with K in Table(3-11) (fig.3-22), BuChE, MDA and HbA1c (0.316, 0.363, 0.557 and 0.999) respectively.

Whilst there was negative correlation between HbA1c with Ca, Na ion, GPX, lipoic acid and SOD (-0.263, -0.047, -0.259, -0.419 and -0.536) respectively, and positive correlation between HbA1c with K (fig 3-21), BuChE and MDA (0.322, 0.366 and 0.561) respectively.

This study revealed there was no correlation between FBG with Na ion and beta- catenin as well as there was no correlation between HbA1c with Na ion and beta catenin.

In comparison with other studies, Rajagambeeram et al., (2020) which found correlation between FBS, HbA1c and serum electrolytes, although the correlations are not significant for both FBS and HbA1c, sodium is negatively correlated with both FBS and HbA1c. Potassium is negatively correlated with HbA1c alone [174].

The current study agreed with trail conducted by Shridhar et al., (2020) that reported a negative correlation was observed between serum Na ion (r=-0.342) and Cl ion (r=-0.538) with random blood sugar. In group 2, a significant correlation was present between serum K^+ and random blood sugar (r=0.356, p) [175], as well agreed with study reported by SAITO *et al*., (1999) who found a negative correlation between serum Na ion levels and fasting plasma glucose (FPG), and positive correlation between serum K^+ levels and FPG. The disorder may be based on the excessive shifting of electrolytes across the cell membrane, dependent on the impaired insulin action as well as hyperosmolality [173].

Esawy & Magdy, (2020) reported that LIAS gene expression in the diabetes group showed significant correlations with disease duration, HbA1c, cholesterol and LDL-C [88], while the current study agreed with Abbott et al., (1993) that reported there was a significant correlation between plasma glucose and serum BuChE in the type 2 diabetic population (r = 0.33) [176] .

Present study disagreed with studies that found GPx and SOD had no statistical correlation with HbA1c reported by Saif-Elnasr et al., (2017) while agreed with Baumüller et al., (2015) that reported there was no correlation found between of β-catenin and HbA1c [177] [178].

Table 3.11. Correlation between the biomarkers and the glucose and HbA1c levels of subjects.

Parameter	FBG	HbA1c
Ca ion	-0.260*	-0.263*
K ion	0.316**	0.322**
Na ion	-0.039	-0.047
GPX	-0.269*	-0.259*
Lipoic acid	-0.419**	-0.419**
BuChE	0.363**	0.366**
B-catenin	-0.073	-0.067
MDA	0.557**	0.561**
SOD	-0.531**	-0.536**
FBG	1	0.999**
HbA1c	0.999**	1

*Weak, **strong correlation

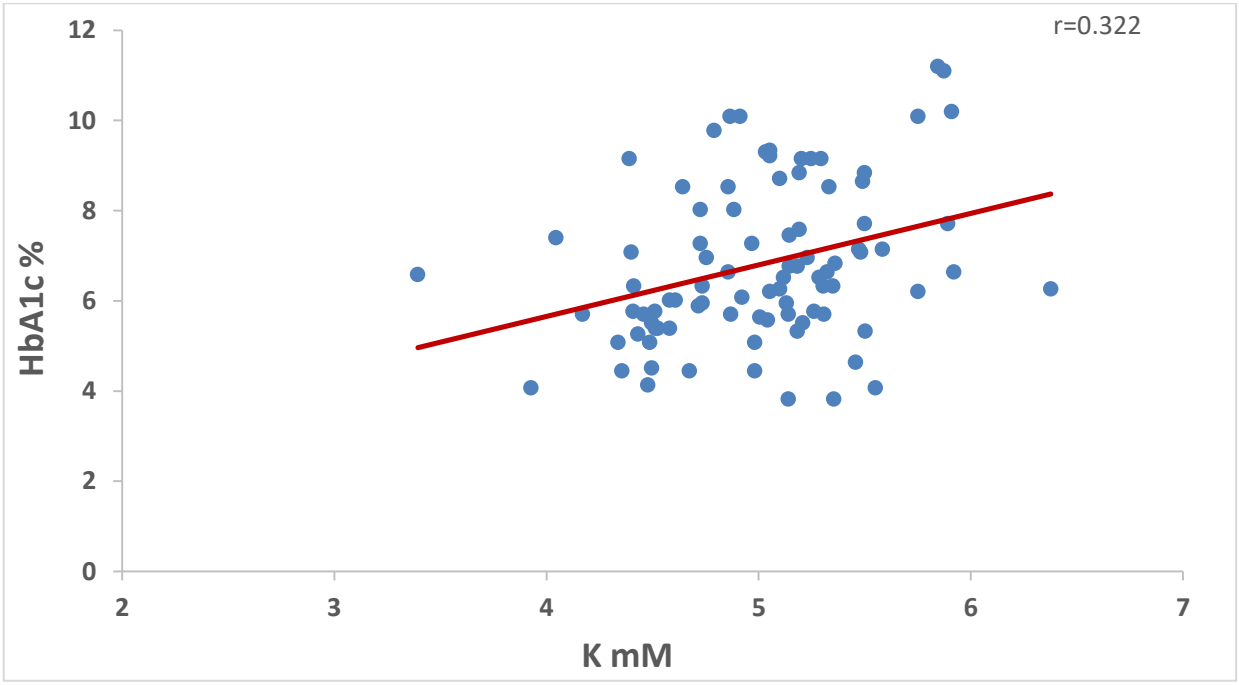


Fig.(3-21): Correlation between serum level of HbA1c and potassium

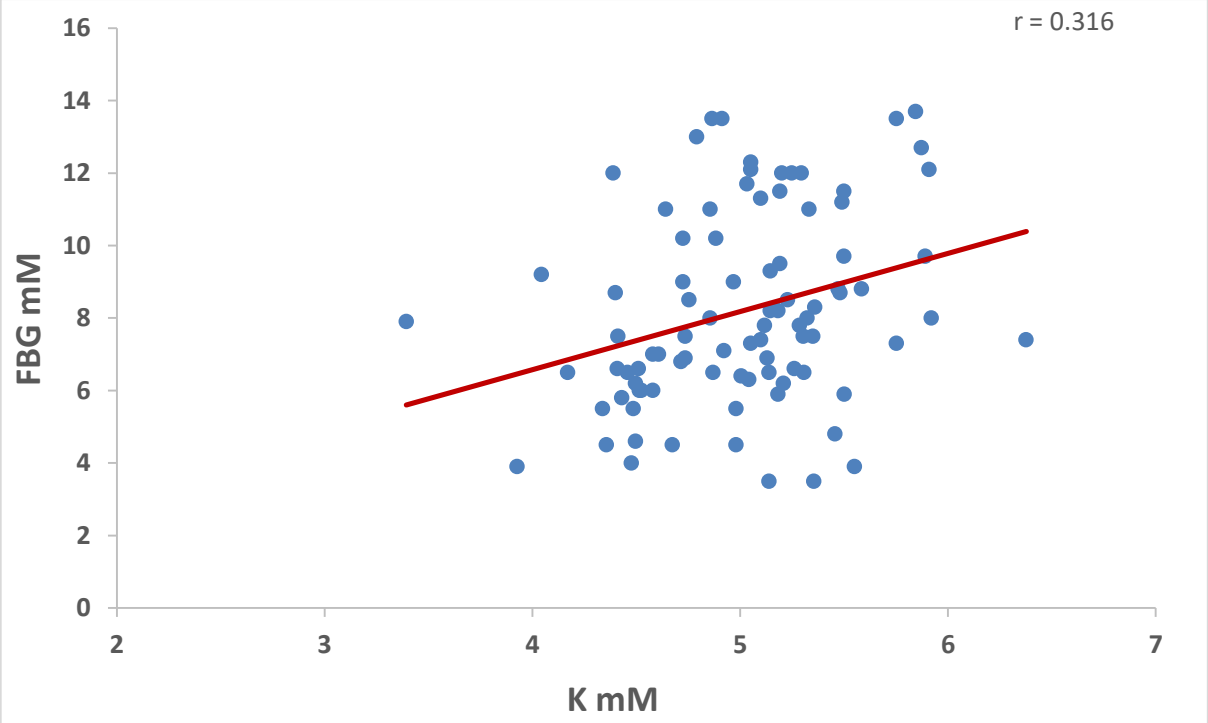


Fig.(3-22): Correlation between serum level of FBG and potassium

3.4. Receiver operating characteristic study

3.4.1. Study of the Parameters for diagnostic characteristics of T2DM

The results in correlation between the biomarkers and Receiver operating characteristic for diagnosis of T2DM are presented in Figure (3.23) and in Table (3.12).

ROC curve was drawn to determine the appropriate cut off value of diabetes and no diabetes participants by using biomarkers of the GPX, SOD, BuChE, beta- catenin and MAD as predicative value on the risk of diabetes that analyzed by ROC curve, and the results are shown in Table(3.12) and Fig(3.23). the cut off level of GPX, lipoic acid, BuChE, beta- catenin, MDA and SOD for diabetes were 51.22, 10.22, 14.24, 5.36, 3.55, 2.15 respectively. The optimal GPX cutoff levels as identified by the maximal Youden index were

51.22 % for diabetes and non-diabetes showed high sensitivity (0.60%) and specificity (0.621%).

The highest sensitivity and specificity analyses were recorded (0.60% of GPX and0.767 % of lipoic acid) and (0.621% of GPX and 0.793%of lipoic acid) respectively, while lowest sensitivity and specificity were recorded to beta catenin (0.533).

The AUCs and 95% CIs of GPX, lipoic acid, BuChE, beta- catenin, MDA and SOD for diabetes were 0.279(0.164-0.393), 0.158 (0.076-0.241), 0.832 (0.924-0.740), 0.514(0.385-0.644), 1.000 (1.000-1.000), 0.016 (0.035-0.000) and 0.016(0.035-0.000) respectively. GPX showed the highest diagnostic values for diabetes other than predictors in every group, MDA and BuChE represented the remarkable increase of the area under the curve (AUC).

In comparison with other studies, once conducted by Fahmy *et al*., (2021) which found GPX as predictive biomarker for DM with shoed higher AUC, optimal cut off level and higher specificity and sensitivity in both control and DM groups that our study agreed with this results while MAD showed high area under curve (AUC) and lower cut off level with high sensitivity this result did agree with our study[179].

Alaaraji *et al*., (2016) used the receiver operator curve (ROC) analysis of the forthcoming variations revealed the descending order of serum MDA (0.999), GSH (0.984) showing a significant variation [180]. Another study by Zhang *et al.,* (2022) performed ROC curve analysis and found the area under the ROC 0.607 (95% CI: 0.52–0.69, p = 0.044) for GPx4 [181]. Therefore, GPx4 exhibited an acceptable capacity to distinguish the GDM (gastric diabetes mellitus) patients from the general population. Over all previous studies demonstrate the possibility of GPX and MDA as biomarkers for the early prediction of DM, these two variables recorded high ROC AUCs and

satisfactory specificity and sensitivity. the study shows high levels of MDA in diabetic patients indicating to an increased oxidative stress which is predictive to DM.

Table (3.12): Diagnostic value of biomarkers (GPX, Lipoic acid, BuChE, B-catenin, SOD and MDA)

Variable	Cut-off Level	Sensitivity %	Specificity %	Youden's J Statistics	AUC	95% CI of AUC	p-value
GPX U/ml	51.22	0.60	0.621	-0.38	0.279	0.164-0.393	<0.001
Lipoicacid pg/ml	10.22	0.767	0.793	-0.56	0.158	0.076-0.241	<0.001
BuChE pg/ml	14.24	0.767	0.791	0.60	0.832	0.924-0.740	<0.001
B-catenin ng/ml	5.36	0.533	0.633	0.16	0.514	0.385-0.644	0.824
MDA µM	3.55	1.000	1.000	1.00	1.000	1.000-1.000	<0.001
SOD U/ml	2.15	0.933	0.957	-0.82	0.016	0.035-0.000	<0.001

AUC: Area under curve. CI: Confidence interval

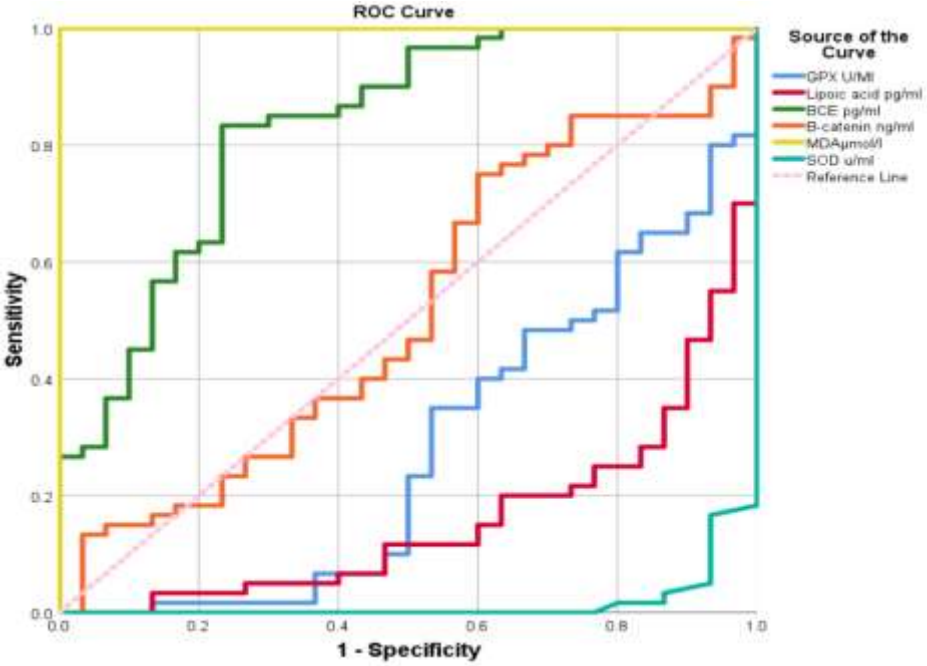
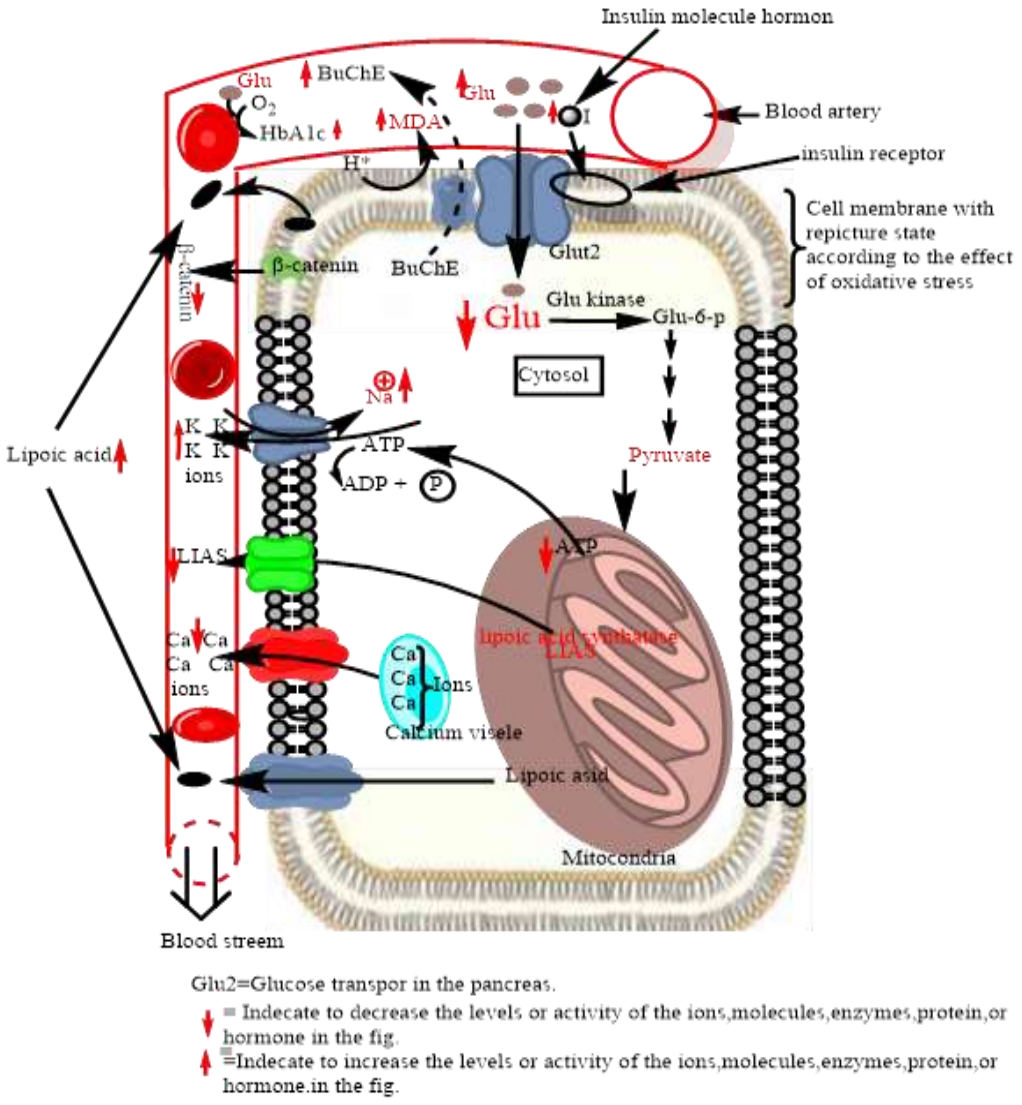


Fig.(3.23) Receiver operating characteristic-area under curve analysis of the measured biomarkers for diagnosis of T2DM from healthy controls

The following diagram shows the relationship between the change in the levels and activities of the enzymes that represent the variables in this study in the serum as a result of the effect of type 2 diabetes on the deformation of the cell membrane due to the high level of oxidants stress.



Glu2=Glucose transpor in the pancreas.

= Indecate to decrease the levels or activity of the ions,molecules,enzymes,protein,or hormone in the fig.

=Indecate to increase the levels or activity of the ions,molecules,enzymes,protein,or hormone.in the fig.

Fig (3.24): Diagram shows the relationship between change in the level of activities of the enzyme of the variables in the serum on the effect of type 2 diabetes due to high level of oxidative stress.

Conclusions and Recommendation

Conclusions

1. The concentration of BuChE enzyme is measured in patients with type II diabetes and the control group, where an increase in concentration levels BuChE is found in diabetic patients compared to the control group and significant association between hyperglycemia and BuChE and higher level of BuChE among patient group ($P< 0.001$).

2. The concentration of β-Catenin is measured in patients with type II diabetes and the control group significant, decrease ($p<0.001$) in β - Catenin level in patient comparison with the healthy control group .No correlation found between beta catenin and other parameter (GPX,SOD,MDA, Na, k, Ca, LIAS, LA, and BuChE).

3. The results show that there was no association between BuChE enzyme

and beta-catenin.

4. The results show a decrease in the activity of the antioxidant enzymes SOD and GPX, and the decrease was statistically significant. The results of the study also showed a high concentration of MDA as a sign of oxidative damage.The results show that there is a correlation and a negative relationship between BuChE enzyme and the activity of SOD enzyme and LIAS, while there is apositive relationship between the enzyme with MDA and k. Negative correlation between MDA with SOD and positive correlation between MDA with Ca and K, as well as there was strong positive correlation between SOD and Ca and negative between K with Na. Positive correlation between lipoic acid synthase (LIAS) and lipoic acid.

5.Negative correlation between FBG with Ca, Na, GPX, lipoic acid and SOD and positive correlation between FBG with K and HbA1c with BuChE and MDA.

6. The highest sensitivity and specificity analyses were recorded (0.60% of GPX and 0.767 % of lipoic acid) and (0.62% of GPX and 0.793%) respectively, while lowest sensitivity and specificity were recorded to beta catenin (0.533). As well as the optimal GPX cutoff levels as identified by the maximal Youden index were 551.22 % for diabetes and non-diabetes.

Recommendations

1.Studying the genetic relationship between BuChE enzyme and B-catenin

in type 2 diabetes mellitus.

2.Studying the relationship between Na/K ATPase enzyme with BuchE

enzyme, beta-catenin and Lipoic acid in patients with type 2 diabetes mellitus.

3. More investigation is needed to reveal the relationship between serum butyrylcholinesterase and diabetes, as well as the physiological function of serum butyrylcholinesterase. Also more researches need to reveal the relationship between serum beta-catenin and diabetes, as well as the physiological function of serum.

Chapter four

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Appendix

Appendix

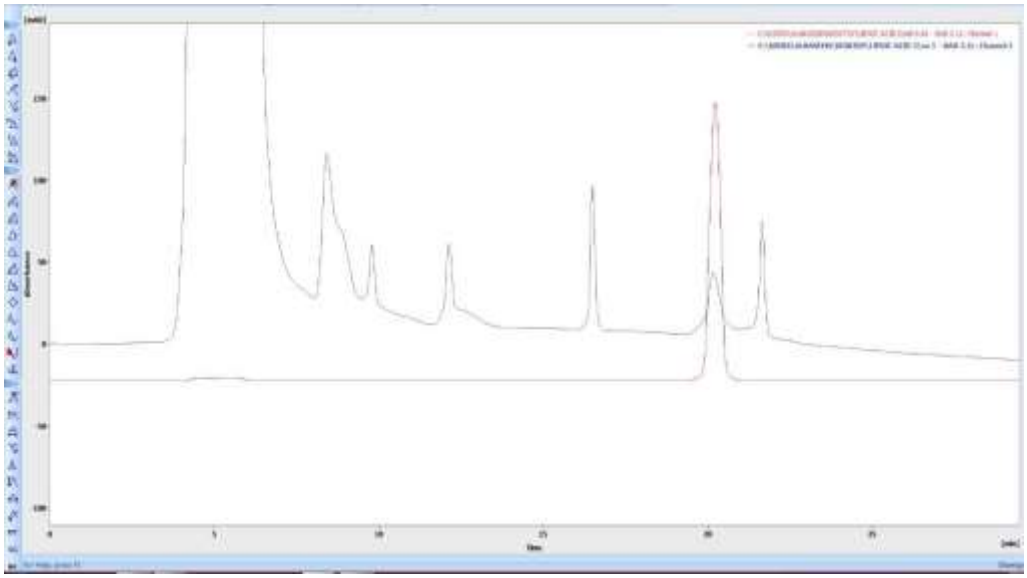

Fig (1): Chromatograms of Lipoic acid of patient with diabetes mellitus type 2

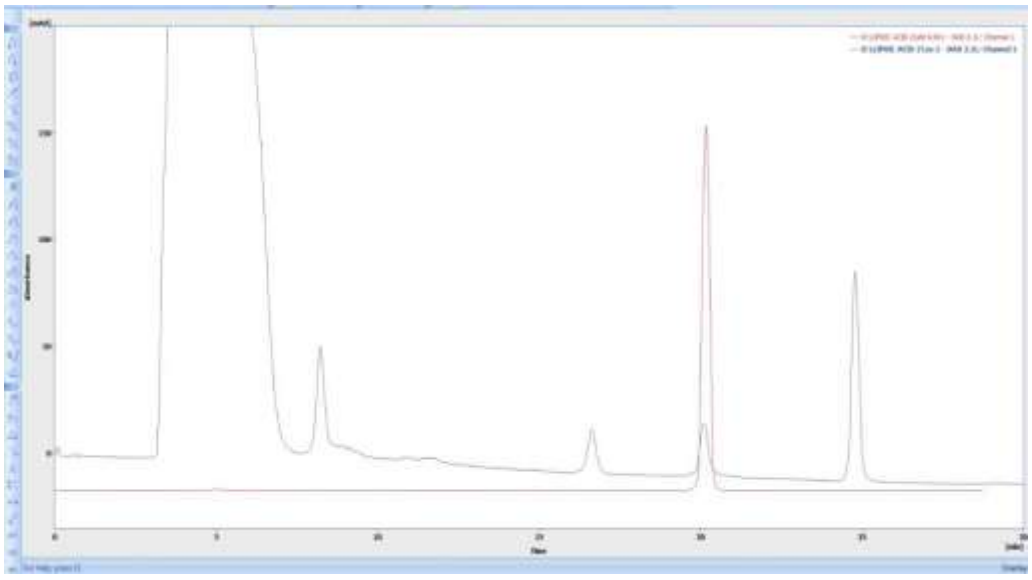

Fig (2): Chromatograms of Lipoic acid patient with diabetes mellitus type 2

Appendix

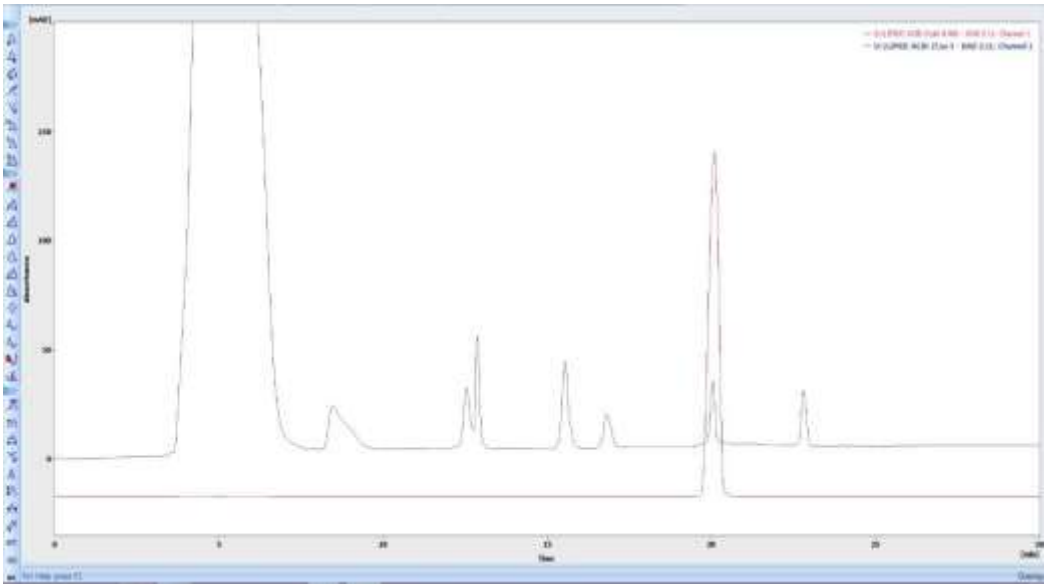

Fig (3): Chromatograms of Lipoic acid of patient with diabetes mellitus type 2

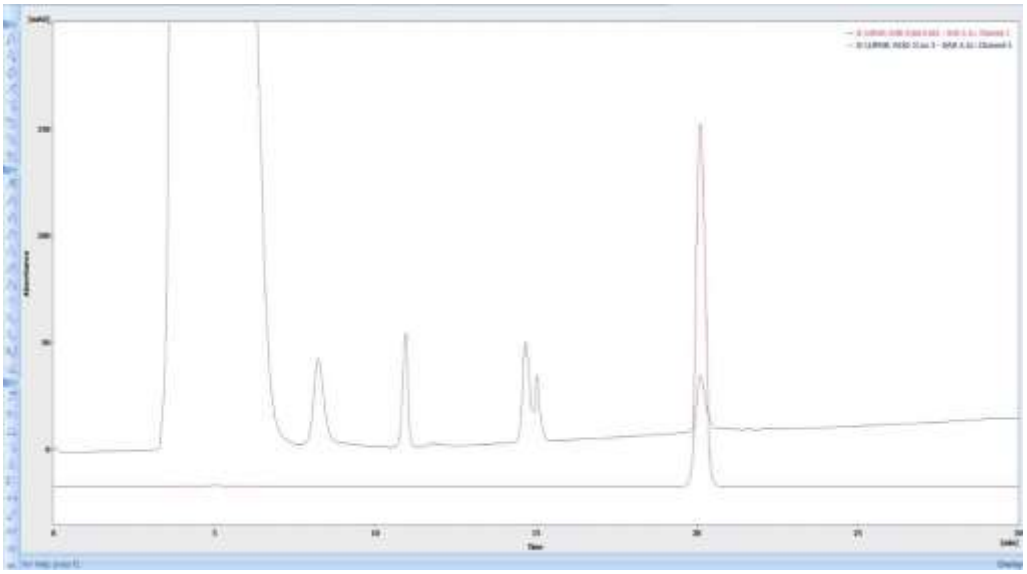

Fig (4): Chromatograms of Lipoic acid of patient with diabetes mellitus type 2

Appendix

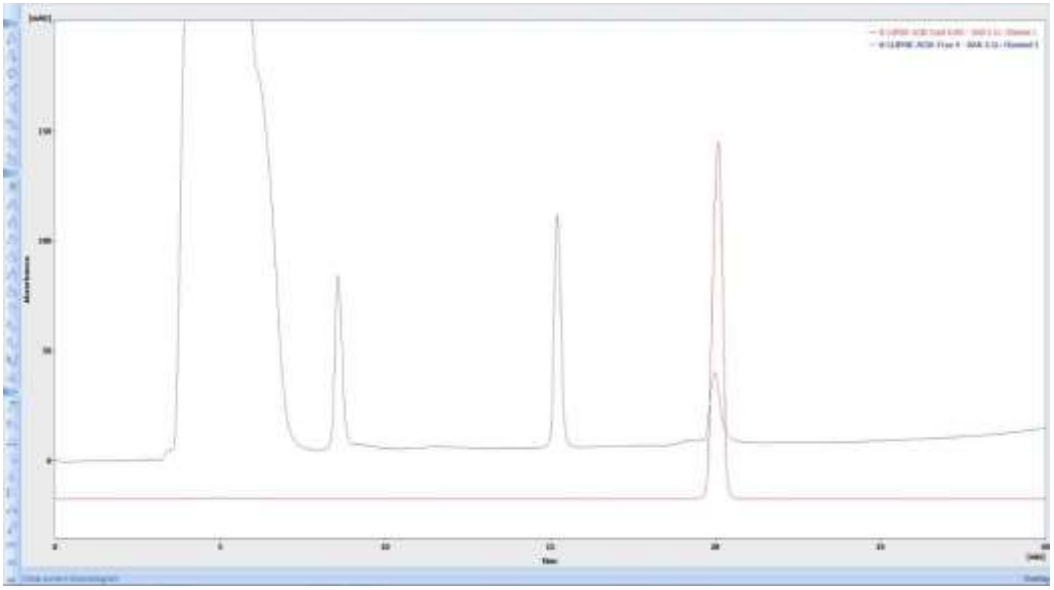

Fig (5): Chromatograms of Lipoic acid of a patient with diabetes mellitus type 2

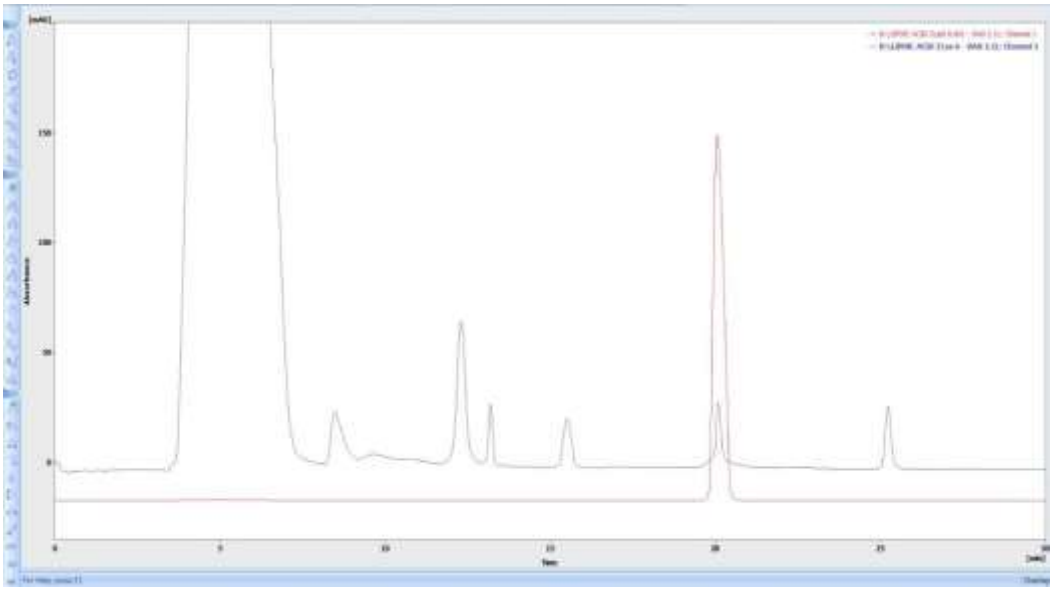

Fig (6): Chromatograms of Lipoic acid of Chromatograms of Lipoic acid helthy volunteer

Appendix

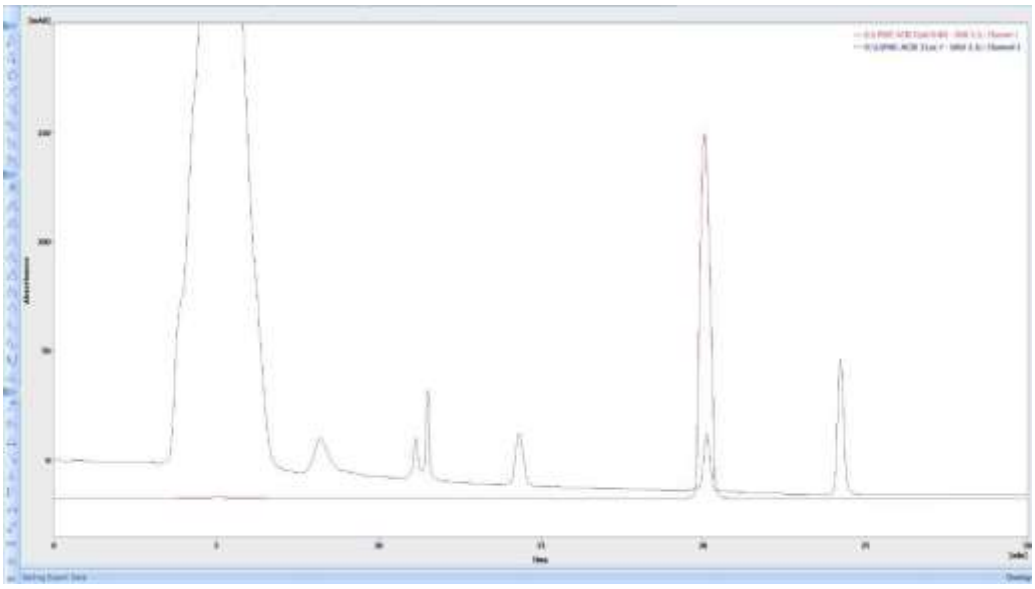

Fig (7): Chromatograms of Lipoic acid of Chromatograms of Lipoic acid healthy volunteer

الخلاصة

داء السكري هو أضطراب استقلابي ناتج عن خلل في افراز الانسولين أو كليهما يؤدي نقص الأنسولين بدوره الى فرط سكر الدم المزمن مع اضطرابات التمثيل الغذائي للكربوهيدرات والدهون والبروتين ،وتهدف هذه الدراسة الي إيجاد ارتباط بين فعاليه البيوترايل كولين استريز والبيتا - كاتنين مع بعض المتغيرات الحيويه في مرضى السكري النوع الثاني.

تصممت هذه الدراسة بدراسة المرضى والاصحاء حيث تألفت من 60 مريضا بالسكري من النوع الثاني و30 من غير المرضى(أختيرت من مركز السكري والغدد الصماء في منطقه الحلة ، بابل ،العراق، مستشفى مرجان التعليمي).المجموعات متطابقة من حيث العمر و أستخدام المصل serum كنماذج للدراسة. من اجل قياس(جلكوز الدم الصائم) HbA1c،FBG، مالونديالديهايد (MDA)،الجلوتاثيون بيروكسيديز (GPX)،سوبراكسيد ديسميوتاز(SOD)،حمض ليبويك(LA)،انزيم تخليق حمض الليبويك(LIAS)،بوتريل كولينستراز(BuChE) ،بيتا –كاتنين والكتروليتات(Ca،k،Na).

وجدت النتائج ان النسبة الأكبر من عينة الدراسة المرضى الذين يعانون من ارتفاع السكر في الدم من النوع الثاني مرتبط بارتفاع مؤشر كتلة الجسم وليس هناك ارتباط معنوي بين العمر والجنس، حيث يؤدي ارتفاع السكر في الدم الى انخفاض GPX، SODوزياده في MDA بفعل اكسدة الدهون، وهذا يدل على زياده الاجهاد التأكسدي الحاصل بفعل زيادة الجذور الحرة.

هناك ارتباط كبير بين ارتفاع السكر في الدم وBuChE،فضلا عن وجود ارتباط معنوي بيت ارتفاع السكر في الدم وانزيم تخليق حمض الليبويك LIASولم يكن هناك ارتباط بين ارتفاع السكر في الدم وحمض ليبويك.

بينما لا يوجد ارتباط بين ارتفاع السكر في الدم والبيتا كاتنين،وارتباط كبير بين ارتفاع السكر في الدم والكالسيوم مقارنة بالمجموعة الصحية ،بينما لا يوجدارتباط بين ارتفاع اليكر في الدم وبيتا - كاتنين وارتباط كبير بين السكر في الدم والكالسيوم في المرضى الذين يعانون من انخفاض الكالسيوم مقارنة بالمجموعة السليمة.

لا يوجد ارتباط كبير بين الكتروليتات في المصل (Ca،k،Na) ومضادات الاكسدة (MDA،GPX،SOD) لمرضىHbA1c الجيد والغير جيد، بينما كان هناك ارتباط معنوي(P> 0.001)بين FBGوHbA1c لكل منHbA1c الجيد والسيئ.علاقة إيجابية قوية بين GPXو انزيم تخليق حمض ليبويك وايون الكالسيوم والعلاقه عكسية ضعيفة مع MDA،بينما هناك علاقة إيجابية بين BuChE وMDA و K ايون وعلاقة سلبيه بينBuChE و SOD ولم يتم العثور على ارتباط بين البيتا كاتنين والعلامات الحيويه الأخرى.

علاقة سلبية بينMDAو SODوعلاقة موجبة بين MDA و Ca، k ايون ،وكذلك كان هناك علاقة موجبة قوية بين Ca،SOD أيون و العلاقة السلبية بينk ايون مع Na ايون تم تسجيل اعلى تحليلات حساسية ونوعية (0.60% من GPXو0.79.3% من حامض ليبويك) و(0.60% من GPX،0.767% من حامض الليبويك) على التوالي بينما سجلت اقل حساسية ونوعية لبيتا كاتنين(0.533)بالإضافة الى مستويات القطع المثلى ل GPX كما حددها مؤشر Youden الأقصى كانت 551.22% لمرض السكري وغير السكري.

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تقدير نشاط بيتا- كاتنين والبيوتيريل كولين استريز في أمصال دم مرضى السكري من النوع الثاني

رسالة مقدمة الى

مجلس كلية العلوم-جامعه بابل

كجزء من متطلبات نيل درجه الماجستير في

علوم الكيمياء

من قبل

صابرين صالح جابرحسين الخفاجي

بكالوريوس علوم كيمياء/ جامعه بابل(2008)

باشراف

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