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Role of Mitochondrial DNA, Chromium Ion and biochemical parameters in Pathogenesis of Type 2 Diabetes Mellitus with and without Ischemic Heart Diseases

A Thesis

Submitted to the Council of College of Medicine, University of Kerbala, in Partial Fulfillment of the Requirements for the Degree of Master in Clinical chemistry

By

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بسم الله الرحمن الرحيم

﴿قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ﴾

صدق الله العَلِيُّ العَظِيمُ

سورة البقرة / الآية ٣٢

Dedication

To the sparks that kept me going:

My Father´s Soul

My Mother

My wife

My Brother and Sisters.

Finally to My Sons

Abdullah and Faisal

Ali

Acknowledgments

In the name of God, the most merciful and most gracious.

First, and above all, I praise God, the almighty, for providing me this opportunity and granting me the capability to proceed to this stage.
After that, I would like to thank all the patients who have cooperated with me and also the patients' families. I wish God to write to them healing and wellness.
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Supervisor's Certification

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"Role of Mitochondrial DNA, Chromium Ion and Biochemical Parameters in Pathogenesis of Type 2 Diabetes Mellitus with /without Ischemic Heart Diseases "

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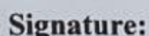
Signature: 
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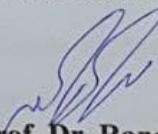

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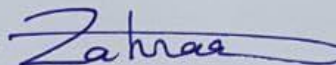
Signature: 
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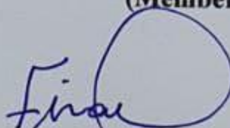
Signature: 
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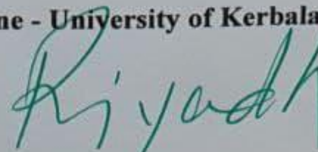
Signature: 
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II

Summary

Diabetes mellitus (DM) is a metabolic disorder characterized by the presence of chronic hyperglycemia accompanied by greater or lesser impairment in the metabolism of carbohydrates, lipids and proteins.

One of important complications of DM is ischemic heart disease, therefore this cross-sectional study attempt to study the correlation and associations by many variant biomarkers and the specific mutation compared to patients without ischemic heart disease. The study select two study groups, first group include type 2 diabetes mellitus type 2 with ischemic heart disease (50 patient), and the second group of the presented by type 2 diabetes mellitus without ischemic heart disease (50 patients).

By using EDTA tube the blood sample was withdrawn from each patient, then mitDNA was extracted by specified kit. Then the extracted mitDNA from sera of type 2 diabetes mellitus with and without ischemic heart diseases was determine by using real time PCR technique to investigate specified mutation at base pair (3243) that occur in mitDNA in order to differentiate between the different diseases.

Our result found a strong positive correlation between HOIR and Insulin (r= 0.926) with significant differences ($P<0.05$) in the blood of patient have ischemic heart disease and the result found mid correlation (0.568) with significant differences ($P < 0.05$) between insulin levels with chromium in blood of patient have ischemic heart disease. When the chromium level is less than 4.25 ppb, the individual is classified as having cardiovascular disease. The element chromium has a potential association with ischemic heart disease and has been designated as a prediction marker.

The amplification of the *MTLL1* gene gives one genotypes as indicated by (422bp) bands for those with homozygous wild type (AA), homozygous mutant (GG) genotypes and two genotypes bands (422 bp) for those with hetrozygouse (GA) .

Genotype frequencies of A3243G polymorphism were found to be consistent with Hardy–Weinberg equilibrium. Allele frequencies (32 %, 58 %, 10 %) of AA, GA, GG in cases of T2DM with ischemic heart disease group and (76 %, 22 %, 2 %) of AA, GA, GG in cases of DM without ischemic heart disease group.

The level of chromium is 12.4460 ppb in sera of ischemic heart disease as compared with that in sera without ischemic heart diseases 4.99 ppb and is significant correlated ($P< 0.05$).

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List of abbreviations

Abbreviations	Description
ALP	Alkaline phosphatase
ASPCR	Allele-specific polymerase chain reaction
WHO	World Health Organization
BMI	Body mass index
GDM	Gestational diabetes mellitus
CEA	Carcino embryonic antigen
CLIA	Chemiluminescence Immunoassay
HIV	Human immunodeficiency virus
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
QUICKI	quantitative insulin sensitivity check index
EDTA	Ethylene diamine tetra acetic acid
EGF	Like growth factor
ER	Estrogen receptor
G	Grade
mitDNA	Mitochondrial Deoxyribonucleic acid
HRT	Hormone replacement therapy

HWE	Hardy–Weinberg equilibrium
ƛ2	Chi- square
LCIS	Lobular in situ carcinoma
LD	Disequilibrium
MMP-2	Matrix metalloproteinase-2
MMPs	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MUC1	Mucin
NPV	Predictive value of a negative
OR	Odd ratio
PCR	Polymerase chain reaction
PPV	Predictive value of a positive
PR	Progesterone receptor
ROC	Receiver-operating characteristics
S	Stage
SD	Stander deviation
SE	Sensitivity
SNPs	Single nucleotide polymorphisms
SP	Specificity
STM	Serum tumor markers
TBE	Tris borate EDTA
TIMP-2	Tissue inhibitor of metalloproteinases2
Anti GAD	Anti-glutamic acid decarboxylase

CHAPTER ONE

Introduction

and

Review of Literature

1. Introduction

Diabetes mellitus, commonly known as diabetes, is a group of metabolic disorders characterized by a high blood sugar level over a prolonged period of time.one of the most important symptoms (polyuria , weight loss , constant thirst), If left untreated, diabetes can cause many complications. Acute complications can include diabetic ketoacidosis, hyperosmolar hyperglycemic state, or death. Serious long-term complications include cardiovascular disease, stroke, chronic kidney disease, foot ulcers, damage to the nerves, damage to the eyes and cognitive impairment.

Generally diabetes mellitus is caused by an absolute or relative insulin deficiency. It has been defined by the World Health Organization (WHO), on the basis of laboratory findings, as a fasting venous plasma glucose concentration of 7.0 mmol/l or more (on more than one occasion or once in the presence of diabetes symptoms) or a random venous plasma glucose concentration of 11.1 mmol/l or more. Sometimes an oral glucose tolerance test (OGTT) may be required to establish the diagnosis in equivocal cases. The interpretation of this test is shown below, but, briefly, diabetes mellitus can be diagnosed if the venous plasma glucose concentration is 7.0 mmol/l or more (fasting) and/or 11.1 mmol/l or more 2 h after the oral ingestion of the equivalent of 75 g of anhydrous glucose. Diabetes mellitus can be classified into various types **(Crook, 2013).**

Type 1 diabetes mellitus previously called insulin-dependent diabetes mellitus (IDDM), type 1 diabetes mellitus is caused by loss of insulin-secreting capacity due to selective autoimmune destruction of the pancreatic beta cells**.** Insulitis (i.e., mononuclear-cell infiltration of the pancreatic islets) is the direct result of the autoimmune process. Antibodies to the cytoplasm of islet cells, glutamic acid decarboxylase, insulin, and tyrosine phosphatase–like protein (IA-2 or IA-2b), which appear before the clinical onset of diabetes, are good markers of the autoimmune process. Several lines of evidence have suggested that autoimmunity

is not the only cause of beta-cell destruction. The American Diabetes Association and the World Health Organization have proposed that type 1 diabetes be subdivided into autoimmune (immune-mediated) diabetes (type 1A) and idiopathic diabetes with beta-cell destruction (type 1B). However, the specific characteristics of the idiopathic subtype are largely unknown **(Imagawa *et al.*, 2000)**.

Mitochondrial DNA comprises 0.1–2% of the total DNA in most mammalian cells. There are several unique features of the mitDNA: human mitDNA is circular, 16 kbp long, and inherited from the mother. It encodes two rRNAs, 22 tRNAs, and 13 proteins, all of which are involved in the oxidative phosphorylation process **(Bibb *et al.*, 1981)**

The intragenic sequence is almost absent or limited to a few bases, and mitDNA does not have histones, instead it is organized in nucleoid structures. A large number of experiments showed that multiple copies of mitDNA could be found in each nucleoid, usually from two to 10 copies each, depending on the cell line studied. However, quantitative analysis of the size and mitDNA content of the nucleoid in cultured mammalian cells suggests that an average nucleoid may contain five to seven mitDNA molecules packed in a space of 70 nm .

The A3243G mutation of the mitochondrial tRNA(Leu) gene was found to segregate with maternally inherited diabetes mellitus, sensorineural deafness, hypertrophic cardiomyopathy, or renal failure in a large pedigree of 35 affected members in four generations. Presenting symptoms almost consistently involved deafness and recurrent attacks of migraine-like headaches, but the clinical course of the disease varied within and across generations. The A3243G mutation has been previously reported in association with the mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episode syndrome (MELAS) and with diabetes mellitus and deafness. To our knowledge, however, hypertrophic cardiomyopathy is not a common feature in people with the A3243G mutation and renal failure has not been hitherto reported in association with this

mutation. The present observation gives additional support to the variable clinical expression of mitDNA mutations in humans .**(Wilson *et al.*, 2004)**

Chromium(III) has been proposed to have a nutritional or pharmacological role in changing body composition and improving symptoms of insulin resistance, type 2 diabetes, and related conditions although the mode of action of Cr(III) at a molecular level has failed to be elucidated **(Heshmati *et al.*, 2018).**

Therefore, chromium malate had beneficial influence on improvement of controlling glucose levels and insulin resistant in L6 cells by regulating proteins production and genes expression in glucose uptake and insulin sensitivity signaling pathways.

1.1. Review of Literature

1.1.1. Diabetic Mellitus

Diabetes mellitus, sometimes known as diabetes, is a set of metabolic diseases defined by a chronically high blood sugar level (hyperglycemia). One of the most significant symptoms (polyuria, weight loss, and continuous thirst), diabetes can lead to a variety of problems if left untreated. Diabetic ketoacidosis, hyperosmolar hyperglycemia, and mortality are all symptoms of acute complications. Cardiovascular disease, stroke, chronic kidney disease, foot ulcers, nerve damage, eye damage, and cognitive impairment have all been serious long-term effects **(Gyamfi *et al.*, 2019)**.

In general, diabetes mellitus is caused by the lack of insulin, either absolute or relative. The World Health Organization (WHO) defines diabetes as a fasting venous plasma glucose concentration of 7.0 mmol/l or greater (on more than one occasion or once in the presence of diabetes symptoms) or a random venous plasma glucose concentration of 11.1 mmol/l or higher. In equivocal instances, an oral glucose tolerance test (OGTT) may be required to establish the diagnosis. This test's interpretation is given below, but in brief, diabetes mellitus is diagnosed when

the venous plasma glucose level is 7.0 mmol/l or higher (fasting) and/or 11.1 mmol/l or higher 2 hours after ingesting the equivalent of 75 g of anhydrous glucose oral. Diabetes mellitus is classified into the following groups **(Crook, 2013):**

1. Type 1 diabetes (due to autoimmune b-cell destruction, usually leading to absolute insulin deficiency)
2. Type 2 diabetes (due to a progressive loss of b-cell insulin secretion frequently on the background of insulin resistance)
3. Gestational diabetes mellitus (GDM) (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 causes, e.g., monogenic diabetes syndromes (such as neonatal diabetes and young-onset diabetes), exocrine pancreas diseases (such as cystic fibrosis and pancreatitis), and drug- or chemical-induced diabetes (such as with glucocorticoid use, in the therapy of HIV/AIDS, or after organ transplantation) **(Care, 2018**).

1.1.1.1. Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) is an autoimmune disease that causes insulin deficiency and hyperglycemia. Over last 25 years, there's been a rapid increase in knowledge about type 1 diabetes, resulting in a wide understanding of many facets of the illness, including its genetics, epidemiology, immunological and -cell phenotypes, and disease burden. Interventions to preserve cells have been tried, as have several methods to improve clinical disease management. However, here are significant gaps in our understanding of type 1 diabetes, as well as our capacity to standardize clinical care and reduce illness complications and burden.

Type 1 diabetes mellitus was previously known as insulin-dependent diabetes mellitus or juvenile-onset diabetes mellitus. It is a chronic illness characterized by insulin deficiency production in the pancreas. Type 1 diabetes mellitus is a common

type of the illness in children and adolescents, accounting for 5–10% of all diabetes cases. It is uncommon throughout the first nine months of life and is most prevalent all around age of 12 years. When a patient is insulin-deficient, glucose cannot reach the body cells that require insulin-mediated glucose uptake. This can result in extremely high blood glucose levels, and diabetic ketoacidosis, that can be deadly. This information suggests that type 1 diabetes causes a more vigorous autoimmune response in young children. Diabetes type 1 is an autoimmune disease in which cytotoxic CD8-T lymphocytes attack and destroy pancreatic islets **(Dedov *et al.*, 2017).** There are several points when we must pause in order to learn certain facts and complications (chronic complications):

- **a.** Type 1 diabetes mellitus (T1DM) is a metabolism disorder caused either by chronic autoimmune destruction of insulin-producing pancreatic beta cells.
- **b.** T1DM has become more common throughout the world at a pace of 3% to 5% per year.
- **c.** Regardless of the fact that the disorder is clearly polygenic (approximately 40 loci impacting susceptibility have been identified to date), the major histocompatibility complex provides and over half of disease susceptibility for T1DM (MHC).
- **d.** T1DM patients' pancreas has an islet immunological infiltration derived from a variety of immunologic phenotypes, is larger and thicker, and also has unusual exocrine properties.
- **e.** The long-held model of T1DM's natural history has been extensively updated, and there is a greater appreciation for the disorder's heterogeneity in pathogenesis and symptomatic presentation.
- **f.** A combination of immunologic, genetic, and metabolic markers of disease could be used to predict disease risk.

g. Extensive efforts are being undertaken to prevent and cure the disease, but while there has been progress, there is still no universally accepted method for doing so in a public health care setting.

h. There is a growing recognition that T1DM may be a heterogeneous disorder with a common phenotype at clinical presentation/diagnosis, rather than a singular disease.

i. Significant advances in disease management, achieved by technological advances in insulin analogues and other fields, lay the foundation for significant reductions in hemoglobin A1c (HbA1c) and improved diabetes treatment.

j. Numerous metrics (costs, patient outcomes, etc.) indicate that specialists (endocrinologists) provide more effective support for patients with T1DM than people who do not have T1DM **(Vargatu, 2016).**

1.1.1.2. Type 2 Diabetes Mellitus

As diabetes mellitus is the axis of this research, we will cover a wide range of topics (compilations, symptoms, diagnosis, epidemiology and relationship with mitochondrial DNA).

1.1.1.2.1. Pathogenesis of Type 2 Diabetes Mellitus

Diabetic kidney disease is the leading cause of chronic kidney disease worldwide, reflecting the sharp increase in Type 2 diabetes mellitus prevalence. The presence of (micro)albuminuria has traditionally been shown to diagnose diabetic kidney disease **(Mogensen, 1984).** Microalbuminuria is widely thought to be an early clinical manifestation of diabetic kidney disease, with decreased GFR developing secondarily, especially in people with long-standing diabetic **(Takagi *et al.*, 2015).** So diabetes retinopathy is a frequent eyesight complication of the both type 1 and type 2 diabetes. The inner and outer blood-retinal barriers protect the retina by regulating ion, protein, and water flux into and out of the retina under

physiological conditions. Many causes, including inflammation, contribute to the rupture of the inner and/or outer blood-retinal barrier during retinopathy. This rupture causes macular edema, the leading cause of blindness in diabetes individuals. It has been speculated that under these conditions, retinal pigmented epithelium cells, which form the outer blood-retinal barrier, may be subjected to hyperosmolar stress due to several of mechanisms. In this paper, we discuss the possible causes and consequences of hyperosmolar stress on retinal pigmented epithelial cells during diabetic retinopathy, with a particular emphasis on the intimate interplay between inflammation and hyperosmolar stress, as well as current and upcoming new pharmacotherapies for the treatment of ailments **(Willermain *et al.,* 2018).**

Diabetic cardiomyopathy (DCM), only well complication, has emerged as a key cause of heart failure in the diabetic population. The underlying etiology of DCM is unknown. It is defined as a cardiac dysfunction that develops in diabetic patients independently of other major cardiovascular risk factors such as high blood pressure and coronary artery disease. Several pathogenic factors, such as glucose and lipid toxicity, mitochondrial dysfunction, increased oxidative stress, sustained activation of the renin-angiotensin system (RAS), and altered calcium homeostasis, have indeed been discovered, have been shown to contribute to the structural and functional alterations that characterize diabetic hearts. However, all these pathogenic mechanisms appear to stem from the metabolic inflexibility imposed by insulin resistance or lack of insulin signaling. This results in absolute reliance on fatty acids for the synthesis of ATP and impairment of glucose oxidation. Glucose is then rerouted to other metabolic pathways, with harmful effects on cardiomyocyte function. Here, we discuss the role that impaired cardiac insulin signaling in diabetic or insulin-resistant individuals plays in the onset and progression of DCM **(Zamora and Villena, 2019).**

1.1.1.2.2. Epidemiology of Type 2 Diabetes Mellitus

Globally, the number of people with diabetes mellitus has quadrupled in the past three decades, and diabetes mellitus is the ninth major cause of death. About 1 in 11 adults worldwide now have diabetes mellitus, 90% of whom have type 2 diabetes mellitus (T2DM). Asia is a major area of the rapidly emerging T2DM global epidemic, with China and India the top two epicenters. Although genetic predisposition partly determines individual susceptibility to T2DM, an unhealthy diet and a sedentary lifestyle are important drivers of the current global epidemic; early developmental factors (such as intrauterine exposures) also have a role in susceptibility to T2DM later in life. Many cases of T2DM could be prevented with lifestyle changes, including maintaining a healthy body weight, consuming a healthy diet, staying physically active, not smoking and drinking alcohol in moderation. Most patients with T2DM have at least one complication, and cardiovascular complications are the leading cause of morbidity and mortality in these patients. This Review provides an updated view of the global epidemiology of T2DM, as well as dietary, lifestyle and other risk factors for T2DM and its complications **(Zimmet, 2017).**

1.1.1.2.3. Symptoms of Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is an expanding global health problem, closely linked to the epidemic of obesity. Individuals with T2DM are at high risk for both micro vascular complications (including retinopathy, nephropathy and neuropathy) and macro vascular complications (such as cardiovascular comorbidities), owing to hyperglycemia and individual components of the insulin resistance (metabolic) syndrome **(Krishnan *et al.*, 2018).**

Side effects of T2DM it is in generally gastrointestinal, such as anorexia, nausea, abdominal discomfort and diarrhea; they are usually mild and transient. Also, metformin reduces intestinal absorption of vitamin B12.

Less common is lactic acidosis, is very important because of the high case-fatality rate. Predisposing factors are all situations that predispose to hypoperfusion an hypoxemia (sepsis, heart failure, dehydration, acute or progressive renal impairment) **(Mozaffari *et al.*, 2016).**

1.1.1.2.4. Biochemical Abnormalities of Type 2 Diabetes Mellitus

Type 2 Diabetes Mellitus (T2DM) is one of the most common metabolic disorders worldwide and its development is primarily caused by a combination of two main factors: defective insulin secretion by pancreatic β-cells and the inability of insulin-sensitive tissues to respond to insulin **(Roden and Shulman 2019).** Insulin release and action must exactly meet metabolic demand; hence, the molecular processes involved in insulin production and release, as well as the insulin response in tissues, must be closely controlled. Defects in any of the systems involved might thus result in a metabolic imbalance, which contributes to the pathophysiology of T2DM.

T2DM risk factors are a complex mix of genetic, metabolic, and environmental variables that interact and contribute to the disease's prevalence. Although individual propensity to T2DM owing to non-modifiable risk variables (ethnicity and family history/genetic predisposition) has a solid genetic basis, epidemiological research shows that many instances of T2DM can be avoided by changing the key modifiable risk factors (obesity, low physical activity and an unhealthy diet) **(Hu, Manson *et al.* 2001).** The likelihood of having T2DM is heavily influenced by genetic predisposition. Several T2DM genome-wide association studies conducted over the last decade have revealed the complicated polygenic character of T2DM, with the majority of these loci increasing T2DM risk through primary effects on insulin production and a minority acting through lowering insulin action **(McCarthy 2010).**

Obesity (BMI 30 kg/m^2) is the most significant risk factor for T2DM **(Bellou, Belbasis *et al.* 2018)**. It is linked to metabolic imbalances that result in IR **(Sinha 2002).** A sedentary lifestyle is another risk factor for T2DM, as demonstrated by the Women's Health Study and the Kuipio Ischemic Heart Disease Risk Factor Study, which found that participants who walked 2–3 hours per week or at least 40 minutes per week reduced their risk of developing T2DM by 34% and 56%, respectively **(Weinstein, Sesso *et al*. 2004**).

Physical exercise has three key benefits in terms of delaying the onset of T2DM. First, skeletal muscle cell contraction increases blood flow into the muscle, boosting glucose absorption from plasma. Second, physical exercise lowers the dreaded intra-abdominal fat, which is a proven risk factor for IR. Finally, it has been demonstrated that moderate-intensity exercise improves glucose uptake by 40%. Physical exercise enhances glucose uptake and insulin sensitivity, but it can also alleviate or reverse inflammation and oxidative stress, both of which are risk factors for T2DM **(Venkatasamy, Pericherla *et al.* 2013).**

In terms of the disease's pathogenesis, a breakdown of the feedback loops between insulin action and insulin production results in excessively high blood glucose levels. Hyperglycaemia induces an excess of ROS generation by mitochondria, which gives rise to diabetes complications, Even if hyperglycemia is managed, this condition may continue. The damage caused by hyperglycemia-induced oxidative stress can be avoided if excellent glycemic control is established early, but it is difficult to reverse if poor control is maintained for an extended period of time (**Kowluru, Kanwar *et al.* 2007**). There is a link between hyperglycemia, increased oxidative stress, and excessive AGE generation in the early phases of T2DM. As the illness continues, there is chronic protein glycation of respiratory chain components, which, when combined with mitochondrial DNA damage, can result in a hyperglycemia-independent cascade of events, resulting in a synergy between oxidative stress and AGEs **(Ceriello, Ihnat *et al*. 2009).** The

consequences of this metabolic imbalance trigger inflammatory processes by receptor binding of AGEs or ROS, which can alter the content and structure of the extracellular matrix**,** These structural alterations may result in endothelial dysfunction and, as a result, atherosclerosis **(Reddy, Zhang *et al.* 2015).**

Finally, it has been demonstrated that low-grade inflammation, which is implicated in the development of T2DM and associated vascular consequences, mediates metabolic memory. Many environmental variables that promote T2DM development (age, obesity, sedentarism, and diet) cause an inflammatory response that leads to IR and endothelial dysfunction. Obesity activates NF-B, which regulates the expression of inflammatory genes and increases monocyte binding to endothelium and vascular smooth muscle cells, increasing monocyte-to-macrophage development **(Reddy and Natarajan 2011).** Furthermore, NF-B activation promotes the production of inflammatory cytokines implicated in vascular inflammation, as well as the production of endothelial adhesion molecules, proteases, and other mediators. Another important factor that links inflammation and oxidative stress in obesity condition is the Toll-like receptor, which contributes to hypertension, insulin resistance, and obesity **(Guarner and Rubio-Ruiz, 2015).**

T2DM is a diverse and progressive illness characterized by hyperglycemia and induced by abnormalities in insulin production and/or insulin action as a result of a complicated network of clinical circumstances. There are numerous pathways, each driven by different genetic and environmental factors that interact and mutually reinforce each other, increasing the risk of other diseases such as heart disease, peripheral arterial and cerebrovascular disease, obesity, and nonalcoholic fatty liver disease, among others.

1.1.1.2.5. Diagnosis of Type 2 Diabetes Mellitus

A. **Fasting plasma glucose test**: To perform this test, diabetic patients should be fasting at least 8 hours before doing it. When the level of glucose in plasma

more than 110 mg/dl diagnosed as diabetes. Normal value of FBG test (70-110 mg/dl) **(Botnariu, Forna *et* al. 2017).**

B. **Random plasma glucose:** This test does not want fasting individuals. when the level of glucose in plasma(200mg/dl) or more ,the patient is considered as diabetes **(Arsad, Rochmawati *et* al. 2020).**

C. **Urinary glucose**: Glycosuria is predicted if plasma glucose levels above the renal threshold of 180 mg/dl. Glycosuria is defined as a high concentration of glucose in the urine. Under normal physiological conditions, proximal tubular cells reabsorb the majority of glucose in the glomerular filtrate; nevertheless, when plasma and glomerular filtrate glucose concentrations surpass 180 mg/dl, glucose will be expelled in urine **(Théry, Witwer *et* al. 2018).**

D. The glucose tolerance test measured the body's capacity to manage glucose. In the test, a person fasts for at least 8 hours but no more than 16 hours before testing their fasting plasma glucose. Following this test, the individual is given 75 gm of glucose (100 gm of glucose for pregnant women), which is normally in the form of a sweet-tasting beverage that the person drinks. Blood samples are collected three times during fasting to test blood glucose levels, once at the first hour and once at the second hour.

In normal persons, FPG within 70–110 mg/dl, following glucose intake, glucose level rises and reaches at peak within 1 h and then come to normal fasting levels within 1.5–2 h because of normal insulin action and secretion. After 2 h it should be below 120 mg/dl and negative test for glucose in urine. In diabetic patients, the FPG level is substantially higher than normal, and the glucose level rises after 1 hour of glucose consumption and does not return to normal even after 2 hours due to decreased insulin production and action. Glycosuria is the presence of glucose in the urine.

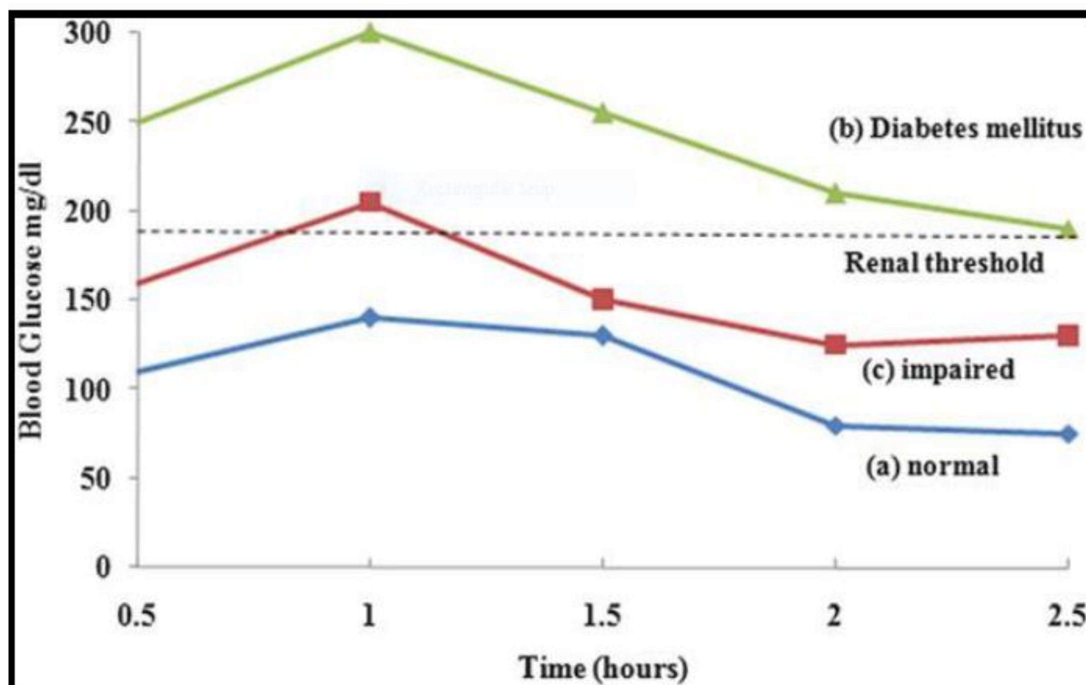


Fig. 1-1: Glucose Tolerance Test

E. Hemoglobin A1c (HbA1c%): The enzymatic attachment of glucose to a protein is known as "glycosylation," whereas the non-enzymatic process is known as "glycation".HbA1c is also known as "glycated hemoglobin" since it is created by non-enzymatic glycation of hemoglobin in which glucose is linked to the N-terminal valine residue of each B-chain of hemoglobin A based on the life period of erythrocytes of 120 days, the HbA1c test is the best measure for monitoring blood glucose levels in diabetic patients over 8-12 weeks, therefore low readings may be detected in individuals with hemolytic anemia. It is more accurate than measuring blood glucose levels since it is unaffected by hormones, food intake, or activity. It is given as a percentage, and the normal level of HbA1c is less than 6%. The number 6% implies very good diabetes control; 7% denotes good control; 8% denotes poor control; and 9% denotes extremely bad control **(Simons, Gordon *et* al. 2016).**

F. Insulin resistance evaluation measuring of insulin resistance is very important for diabetic patient; the homeostasis model assessment of insulin resistance (HOMA-IR) index is widely used in medicine for clinical studies. HOMA-IR

uses fasting measurements of blood glucose and insulin concentrations to calculate indices of both insulin sensitivity and β-cell function. The model assumes that normal-weight subjects aged less than 35 years have an insulin resistance (R) of 1 and 100% β-cell function **(Bakhshalizadeh, Hashemi *et al.* 2016).**

G. C-Peptide: C-peptide is a beneficial and widely used procedure of evaluation pancreatic beta cell function .It is produced in equimolar amounts to endogenous insulin but is secreted at a more stable rate over a longer time. Methods of estimation include urinary and serum sampling. After cleavage of pro-insulin, insulin and the 31-amino-acid peptide" c-peptide" are generate in equal amounts. The degeneration rate of c-peptide is slower than that of insulin (half-life of 20–30min, while the half-life of insulin 3–5 min). In healthy individuals the fasting plasma level of c-peptide is 0.3–0.6 nmol/l, while a postprandial level rise to 1–3 nmol/l. Insulin is metabolized in the liver by first-pass metabolism, whereas c-peptide has small hepatic clearance **(Hellman, Hoffmann *et al.* 2017)**.

C-peptide test use to differentiate T1DM from T2DM (in Type 1 diabetic patients both insulin and c-peptide levels decrease due to β-cell damage while in Type 2 diabetic patients β-cells are there but there is a peripheral resistance to the action of insulin and β-cells trying to produce extra amounts of insulin so insulin level will be normal or slightly elevated and C-peptide level will be high). C-peptide is also used to differentiate hypoglycemia if it is due to exogenous insulin (over dose of insulin therapy) or endogenous insulin (insulinoma).

1.1.1.2.6. Treatment of Type 2 Diabetes Mellitus

Type 2 diabetes mellitus is a chronically progressive disease that is characterized by inherited and acquired insulin resistance and an increasing insulin secretion disorder. In order to reduce the morbidity and mortality rates among type

2 diabetics, which are increased significantly by macro- and micro-angiopathic complications, besides the antihyper glycaemic therapy discussed here it is also essential to administer the optimum treatment for arterial hypertension, diabetic dyslipidaemia and hyper-coagulopathy that are often associated with type 2 diabetes. The effectiveness of a multifactorial intervention in reducing macro- and microvascular complications, evidence class and also mortality (absolute risk lowered by 20% in 13.3 years) **(Garber, Klein *et al.* 2006)** Because type 2 diabetes is a persistently progressing illness, antihyperglycemic medication must be chosen based on the pathophysiological stage of the disease at the time treatment is initiated. Simultaneously, non-pharmacological interventions (organized patient education, nutrition therapy, and exercise therapy are critical throughout the disease's progression. The goal range for HbA1c indicated in this recommendation is 6.5 percent, based on data from the UKPDS, the UKPDS 10-year follow-up, and ADVANCE, and in agreement with the European Diabetes Policy Group as well as the worldwide IDF guidelines **(Matthaei, Bierwirth *et al.* 2009).**

The antihyperglycemic therapy of type 2 diabetes is monitored in the medium to long term by testing HbA1c, which should be done every three months. Furthermore, blood glucose levels are measured to monitor the medication in the short term. This should be done in conjunction with the treating physician, and should take into account the treatment objectives, the existing metabolic status, current therapy, and other individual, societal, and organizational aspects. In theory, blood glucose self-measurements should be conducted on a frequent basis by the patient if the results have therapeutic consequences in the short, medium, and long term. The frequency with which the patient should check his or her own blood glucose levels is determined by the individual treatment plan, which is developed after considering the treatment aim, the quality and stability of metabolic control, the treatment method, and other considerations. The ROSSO research, for example, found a favorable link between self-testing and illness progression

(Aubert, Bona *et al.* 2016). As a result, self-testing is an important part of the management of type 2 diabetes.

1.1.2. Ischemic Heart Diseases

Ischemic heart disease is a fast rising cause of mortality worldwide. This condition is characterized by an inadequate supply of oxygen inside the cardiac muscles as a result of an imbalance between oxygen supply and demand, as well as a heart disease caused by coronary artery stenosis. Biopharmaceutical-based therapy, such as protein, gene, and cell therapy, has enhanced traditional surgery-based therapy for the treatment of ischemic heart disorders. The standard medical treatment relies on the use of drug-eluting stents, coronary artery bypass graft surgery, and anti-thrombosis medications. Biopharmaceutical-based treatments, such as recombinant protein therapy, gene therapy, and cell transplantation, have been identified as potential techniques for promoting neovascularization and increasing collateral blood flow in the ischemic heart. This study looks at the current state and future of treating ischemic heart disease with traditional medical therapy, biopharmaceutical-based therapy based on proteins, and polymeric hydrogels for therapeutic protein delivery **(Choi, Hwang *et al*. 2009).**

Ischemic heart disease and ischemic stroke are both frequent conditions with comparable pathogenesis based on arteriosclerosis. Arteriosclerosis usually affects the patient all over, putting him at risk for both acute coronary syndrome (ACS) and acute stroke. In both circumstances, there is an abrupt shift in circulation, resulting in reduced blood flow to a portion of the heart or brain. Stroke has been compared as a "heart attack" in the brain on occasion. As a result, it is obvious that ACS and acute stroke share epidemiological data, risk and etiological variables, as well as treatment interventions Furthermore, several studies have shown that coronary artery disease is common among stroke patients, and that chronic coronary artery disease increases the chance of having a stroke **(De Silva, Woon *et al.* 2008)**

Diabetes is also a risk factor for ischemic stroke. Subjects with known diabetes and asymptomatic hyperglycemia had an elevated risk of ischemic stroke in the Honolulu Heart Program, and these correlations were independent of age and other vascular risk factors **(Burchfiel, Curb *et al.* 1994).** Furthermore, a recent study found that glucose intolerance increased the risk of stroke in individuals who had previously had a TIA or mild stroke **(Vermeer, Sandee *et al.* 2006).** However, not all ischemic strokes are equally favored by diabetes: depending on the series, the prevalence of diabetes is 18-32 percent in atherothrombotic infarction, 20-32 percent in lacunar infarction, and slightly less in cardioembolic infarction (8-21%) **(Vemmos, Takis *et al*. 2000).**

HBP is a major risk factor for coronary heart disease. Systolic blood pressure is the modifiable factor that best predicts acute myocardial infarction and mortality, according to the GRACE score **(White and Chew, 2008)** epidemiological data and other epidemiological studies have shown that the incidence of cardiovascular diseases increases incrementally with blood pressure, even when it is within the normal range (45 percent of cardiovascular events reported in the Framingham study occurred with a systolic BP of 140 or lower) **(Kannel and Wolf, 2008).**

1.1.3. Mitochondrial DNA

Mitochondrial DNA comprises 0.1–2% of the total DNA in most mammalian cells. There are several unique features of the mitDNA: human mitDNA is circular, 16 kbp long, and inherited from the mother. It encodes two rRNAs, 22 tRNAs, and 13 proteins, all of which are involved in the oxidative phosphorylation process **(Ballard and Whitlock, 2004).**

The intragenic sequence is almost absent or limited to a few bases **(Ojala *et al.*, 1981)**, and mitDNA does not have histones, instead it is organized in nucleoid structures. A large number of experiments showed that multiple copies of mitDNA

could be found in each nucleoid, usually from two to 10 copies each, depending on the cell line studied **(Iborra *et al.*, 2004).**

Two different strands can be recognized in the mitDNA: the heavy strand rich in guanine bases, which also contain the majority of mitochondrial coding genes, and the light strand, encoding only for the MT-ND6 (NADH-ubiquinone oxidoreductase chain 6) protein and eight tRNAs. Both strands are transcribed at the same time, giving origin to very long transcripts, of almost mitDNA length, that are subsequently processed. Transcription seems to take place in the nucleoids due to the presence of the mitochondrial transcription machinery. However, experiments performed with 5-bromouridine (BrU) aimed at tracking the progress of the nascent RNA transcripts showed that newly transcribed mitRNAs are also found in discrete foci situated in close proximity to mitochondrial nucleoids, called mitochondrial RNA granules **(Antonicka *et al.*, 2013).**

The process of mitochondrial transcription termination is still unclear. There is still a debate if MTERF1 is really needed for the termination of all the transcription processes that originate from the three different promoters of the control region. **(Barchiesi and Vascotto 2019)** have shown that knock-down mice for the MTERF1 gene do not have any notable consequence on the phenotype, and the levels of ribosomal and messenger RNA is unaffected. However, biochemical studies have shown that MTERF1 only partially terminates H-strand transcription **(Terzioglu *et al.*, 2013)** whereas transcription in the opposite direction (L-strand transcription) is almost completely blocked.

Many different proteins are involved in the regulation of transcription, such as hormones, nuclear transcription factors, and chromatin remodeling enzymes which are also able to interact with the mitochondrial DNA, and RNA/DNA modifying enzymes. Here we propose a brief overview of the mitDNA transcription regulation operated by these factors.

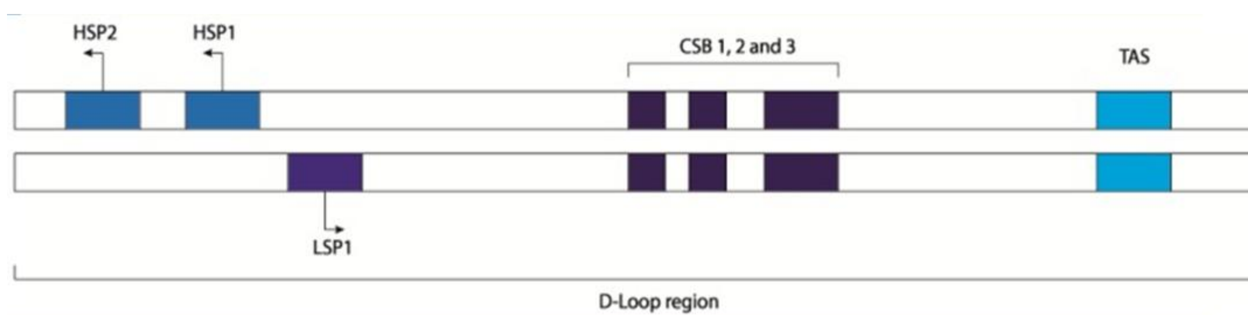


Fig. 1-2: Schematic representation of mitochondrial D-loop region. Heavy strand promoters 1 and 2 (HSP1 and HSP1), light strand promoter 1 (LSP1), conserved sequence blocks 1, 2, and 3 (CSB I, II, and III), and termination-associated sequences (TAS).

One of the first proteins discovered to be involved in the regulation of transcription is the thyroid hormone T3, which is able to promote the mitDNA transcription by directly binding the mitDNA genes **(Psarra and Sekeris, 2008).** Glucocorticoid hormones were also found to be in mitochondria where they modulate the transcription binding to the glucocorticoid receptor present in the mitochondrial inner membrane **(Lapp *et al.*, 2019).**

The estrogen receptor (ER) was found in the mitochondria of cardiac cells. It was hypothesized that E2 (17β-estradiol) and ERβ-mediated cardioprotection was dependent on mitDNA transcription encoding for mitochondrial respiration activity. It was also demonstrated that E2 can also increase the ER β mitDNA binding activity followed by an increase in complex V encoding gene expression **(Hsieh *et al.*, 2006).**

Melatonin was also recently described as a potential hormone that can control the mitDNA expression through the reduction of several mitochondrial transcription factors. It was demonstrated that melatonin was able to decrease, at both mRNA and protein levels, TFAM expression as well as other proteins such as transcription factors TFB1M and TFB2M, interfering with mitDNA transcription **(Hsieh *et al*., 2006).**

1.1.3.1. Biochemical Roles of Mitochondrial DNA

Mitochondria are crucial organelles in eukaryotes, providing critical tasks such as the production of bioenergetic intermediates such as ATP and GTP, as well as the synthesis of nucleotides, Fe-S clusters, heme and amino acids, Fe^{2+}/Ca^{2+} management, inflammation, and apoptosis. Because of their position at such a cellular nexus, mitochondrial malfunction and resultant metabolic abnormalities are implicated in a wide range of human diseases, including both sporadic and familial cancer **(Nunnari and Suomalainen, 2012).** Perturbed cellular metabolism in malignant tissue is a long-standing and well-documented phenomena, with significant research recently revealing distinct routes to mitochondrial malfunction in cancer via mutation or dysregulated expression of nuclear DNA encoding mitochondrial proteins **(Vyas, Zaganjor *et al.* 2016).** The mammalian mitochondrion is made up of 1200 proteins, the great majority of which are encoded in and expressed from the nuclear genome, with just a tiny number encoded by the spatially and heritably distinct mitochondrial genome **(Rhee, Zou *et al.* 2013)**

The OXPHOS system is made up of five multisubunit enzyme complexes that are found on the inner mitochondrial membrane. The mitDNA encodes one or more of the necessary components for the NADH-ubiquinone oxidoreductase (Complex I), ubiquinone-cytochrome c oxidoreductase (Complex III), cytochrome c oxidase (Complex IV), and ATP synthase (Complex V), whereas nDNA encodes the complete succinate-ubiquinone oxidoreductase **(Lott, Leipzig *et al*. 2013 ; Rhee, Zou *et al*. 2013)** the mitDNA strands are known as the heavy strand (H-strand) and the light strand (L-strand), with the former being guanine rich and the latter being cytosine rich. The H-strand encodes 28 genes, whereas the L-strand encodes the remaining nine.

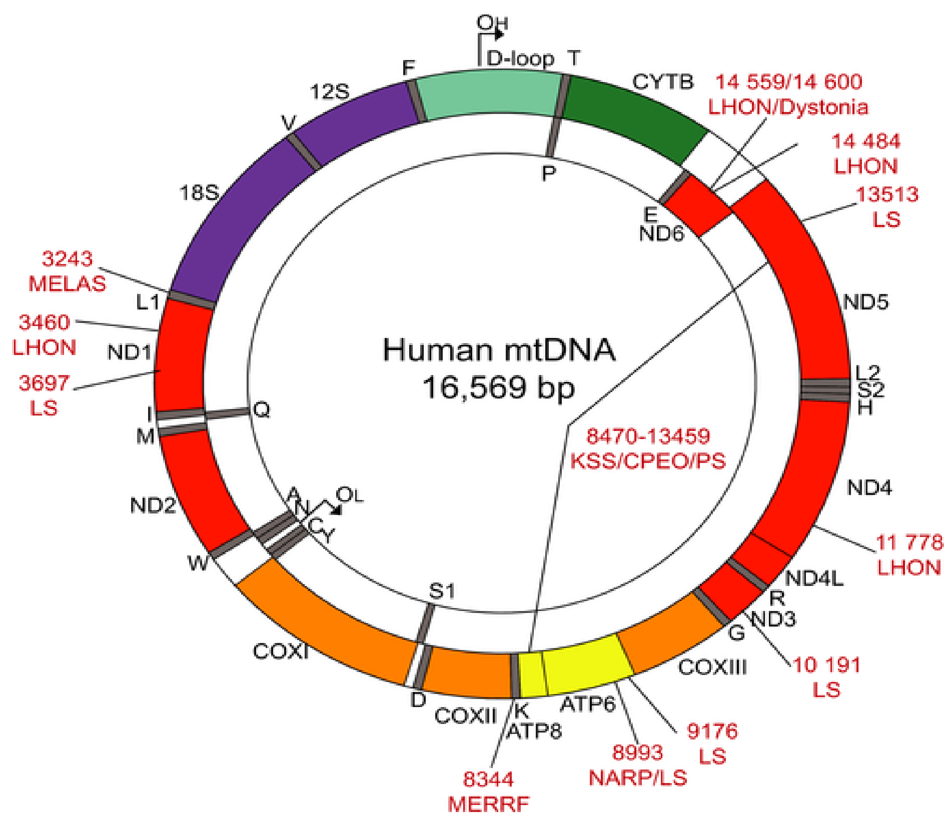


Fig. 1-3: Schematic representation of human mitochondrial DNA

1.1.3.2. Processing of Mitochondrial Transcripts

From their synthesis to their degradation, mitRNAs undergo several stages of maturation and modification for the correct production of mitDNA-encoded proteins. As mitochondrial DNA replication and transcription need to be spatio-temporally regulated to adapt to the metabolic demand of the cell, so must the basic stages of mitochondrial gene expression. To achieve this, mitochondria restrict mitRNA processing and maturation to dynamic protein structures called mitochondrial RNA granules (MRGs), which provide a regulatory function for post-transcriptional processing, allowing all mitRNAs to be fully mature before protein synthesis **(Mai *et al.*, 2017)** characterized the proteome of the granules using GRSF1, a core component of the granule **(Antonicka *et al.*, 2013)** as a bait Some proteins found by the authors were already confirmed in the literature, such as **RNaseP (Jourdain *et al.*, 2013)** and more interestingly the so-called “mitochondrial degradosome” composed of hSUV3 (ATP-dependent RNA

helicase SUPV3L1) and **PNPase** confirming the hypothesis that MRGs are not only sites of RNA processing, but also of RNA degradation and turnover. Mass spectrometry analysis of immune-precipitated fractions showed a large number of proteins responsible for the post-transcriptional processing of the primary polycistronic transcript, such as MRPP (mitochondrial ribonuclease P protein) 1, -2, and -3, RNA-modifying enzymes such as TFB1M (Dimethyladenosine transferase 1), PTCD3 (Pentatricopeptide Repeat Domain 3), and the mitochondrial poly-A polymerase. In addition, proteins belonging to the mitochondrial translation machinery, as well as structural proteins of the small (mt-SSU) and large (mt-LSU) mitochondrial ribosomal subunits, aminoacyl tRNA synthetases, and factors involved in ribosome assembly and disassembly, were present in the analysis. These data suggest that MRGs are also involved in mitochondrial ribosome biogenesis and in mitochondrial translation regulation, with a function analogous to that of the nucleolus, where initial steps of ribosomal assembly are performed (**Tu and Barrientos, 2015**).

It is possible that both mitDNA and its transcription products are portioned within non-membrane bound compartments to provide a greater degree of spatio-temporal regulation of mitRNA processing. The last stage of mitRNA life was suggested to take place in specific foci, called D-foci (degradation foci), composed mostly of the mitochondrial degradosome **(Borowski *et al.*, 2013).**

Mitochondrial DNA transcription takes place between nucleoids and mitochondrial RNA granules (MRGs). TFAM is the principal protein needed for the initiation of transcription as long as POLMRT, TFBM1, and TFBM2 **(Barchiesi and Vascotto 2019).** After transcription of the polycistronic molecules, RNA is immediately processed by RNASE P and Z to release tRNAs, following the tRNA punctuation model. Once tRNAs, mRNAs, and rRNAs are released, the translation process can start **(Doublet, Ubrig *et al.* 2015)** mRNA degradation takes place in the D-foci, close to the MRGs. PNPase and hSuv3 are components of the

degrade some. $REXO_2$ is also present in this compartment and degrades small RNA oligonucleotides (3). Black arrows: transitions; red arrow: inhibition.

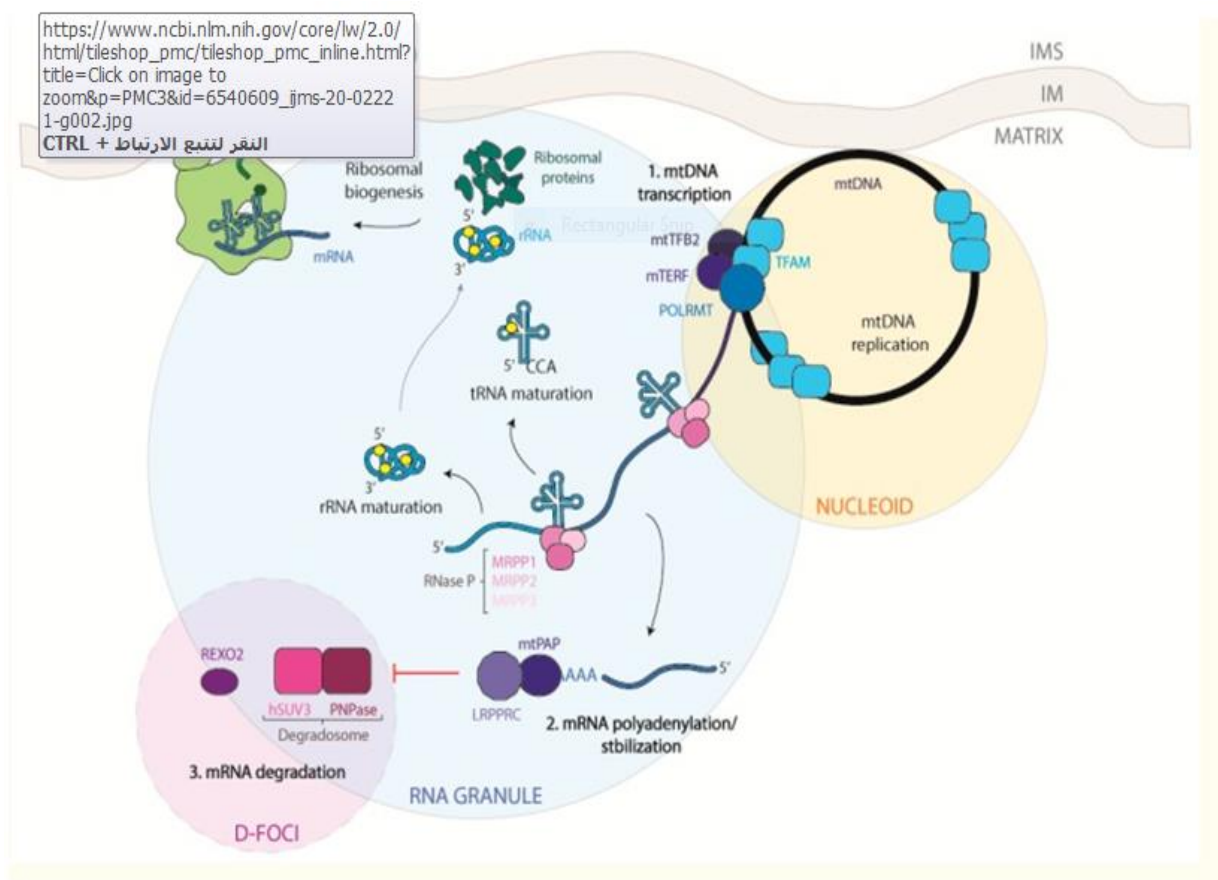


Fig. 1-4: Schematic view of mitochondrial DNA transcription, RNA processing, and degradation.

1.1.3.3. Mitochondrial DNA A3243G Mutation

Human mitochondrial DNA is a circular DNA molecule that encodes some of the proteins required for oxidative phosphorylation. Different mitochondrial DNA genotypes may coexist within a single cell, a condition known as heteroplasmy. An A-to-G transition at position 3243 of mitochondrial DNA(A3243G) can result in maternally inherited diabetes and deafness (mitochondrial diabetes) **(Yan *et al.*, 2014).** The **A3243G** mutation is one of the most common point mutations of the mitochondrial genome (mitDNA). The clinical syndromes that have been historically ascribed to this substitution include mitochondrial encephalopathy with

lactic acidosis and stroke-like episodes (MELAS), maternally inherited deafness and diabetes (MIDD), and progressive external ophthalmoplegia (PEO) **(Mancuso *et al.,* 2014).**

The A3243G mutation is usually present in the heteroplasmic state. The mutation load (cellular content of the A3243G mutation) is dependent on the investigated tissue. It is usually higher in muscle (up to 92%) (38–42) than in hair follicles or hair rods (35%) and higher in hair follicles than in blood lymphocytes. Individual hair roots differ markedly from one another in this respect. The mutation load is usually higher in the buckle (cheek) mucosa (40–55%) than in blood lymphocytes. The mutation may be also detected in skin fibroblasts (50%) or urinary sediments (62%). Other studies found the highest mutation load in the urinary sediment and the lowest in blood lymphocytes. In single pancreatic b-cells the mutation load was low (11–29%) when compared with muscle and brain (66–78%) **(Lynn *et al.*, 2003).** In blood vessels the mutation load may reach 85%. The mutation load reaches 75% in the kidney, 60% in the liver, 58% in the myocardium, and up to 45% in oocytes. In all patients in whom the mutation is detectable in blood it is also detectable in other tissues **(Shanske *et al.*, 2004)**

The high variability in mutation loads between tissues has diagnostic implications, suggesting the skeletal muscle as the most appropriate for genetic analysis. The mutation load increases with age in most tissues. In blood lymphocytes, however, the mutation load decreases with age. This is explained by the preferred selection of cells containing high levels of wild-type mitDNA. The frequency of some phenotypic manifestations of the mutation increases with increasing mutation load, whereas others decrease with increasing mutation load. The higher the mutation load in a mother the more likely she will have an affected child. Generally, the mitDNA copy number is higher in young than in advanced age. A single study also found that the mitDNA copy number decreases with increasing number of affected tissues A high mutation load in the muscle is

associated with SLEs, but one of five patients carrying >80% A3243G remain stroke-free, suggesting additional environmental or genetic factors, such as the background mitDNA sequence variation, to influence the phenotype expression . This has been shown for Leber's hereditary optic neuropathy and for patients with the A3243G mutation. Furthermore, intra-familial clustering of clinical phenotypes in A3243G patients supports a pathogenetic role of the mitDNA background. The risk of SLEs in A3243G patients is increased in the presence of the homoplasmic A12308G polymorphic variant. Together with the polymorphisms A11467G and G12372A the polymorphism A12308G defines the super-haplo group U/K. Haplotype U is also a risk factor for occipital stroke in patients with migraine. An argument against an influence of the mitDNA background on the phenotypic expression, however, is the absence of a relation between the U super-haplotype group and the frequency of SLEs among 107 A3243G patients **(Deschauer *et al.*, 2004).** Furthermore, in a study on 35 A3243G mutants 34 different haplotypes were found, indicating that all instances of the A3243G mutation are most likely caused by independent mutational events. The 34 haplotypes were distributed into 13 haplo-groups of which the frequencies were close to those of the general population, indicating that the A3243G mutation harbors all evolutionary features expected from a deleterious mitDNA mutation under strong negative selection and that mitDNA backgrounds do not play a substantial role in modulating the mutation's phenotype **(Torroni *et al.*, 2003).**

1.1.3.4. Role of Mitochondrial DNA in Clinical Diagnosis

The first mention of mitochondrial DNA depletion illnesses was observed in 1931 in which Alpers' clinical and pathologic condition typically affects young children, creating a severe epileptic syndrome with fast development of dementia

and cortical blindness. Pathologically, Alpers established that this condition mostly affects gray matter **(Yan *et al.*, 2014).**

The first harmful mutations in mitochondrial DNA were discovered in **1988** and it has since become obvious that errors in this small genome are a major cause of neurological illness. However, determining the exact impact of mitDNA illness has been confounded by clinical heterogeneity, the diversity of mutations found across the genome, and the difficulty in making a diagnosis. Advancements in diagnostic tools, as well as the ability to perform extensive family tracing, have led us to reconsider our prior estimates of mitochondrial disease prevalence, with a particular focus on mutations originating in mitochondria **(Chinnery, Johnson *et al.* 2000).**

Individuals who were clinically impacted were those who had symptoms or indications compatible with the molecular genetics. However, this is an uncommon occurrence in the general population. Confirmation of the mitDNA deficiency in each patient was sought but was not needed for inclusion, given the clinical symptoms were consistent and a pathogenic mutation within the pedigree had been proven. This method was validated by a near-100 percent positive rate of genetic testing when samples were available **(Doublet, Ubrig *et al.* 2015).**

Chronic progressive external ophthalmoplegia, cerebellar ataxia, seizures, myoclonus, stroke-like episodes (strokes of thromboembolic origin excluded), proximal weakness, exercise intolerance, cardiomyopathy, optic atrophy, pigmentary retinopathy, or bilateral deafness were all signs of clinically manifest disease **(McFarland, Taylor *et al.* 2002).** Diabetes mellitus was thought to constitute evidence of mitochondrial illness only in pedigrees with mitDNA mutations known to induce reduced glucose tolerance, such as m.3243AG and m.14709TC. **(Man, Turnbull *et al.* 2002).** To establish a cautious minimum prevalence figure, we did not include symptoms such as myalgia, tiredness, migraine, dysphagia, gastrointestinal distress, and cataracts as indicators of

mitDNA illness because they may have significant overlap with prevalent medical disorders. Instead, such individuals were considered "at risk" for developing mitDNA illness, even though the dangerous mitDNA mutation had already been confirmed.

1.1.4. Insulin Hormone

1.1.4.1. Chemistry of Insulin Hormone

Insulin is a protein secreted by the beta islet cells of the pancreas and consists of 2 polypeptide chains with 51 amino acids **(Aloysius *et al.*, 2019)**. In insulin molecule, the chain A consists of 21 amino acid residues and chain B of 30 amino acid residues linked by disulfide bridges (Fig 1-3).

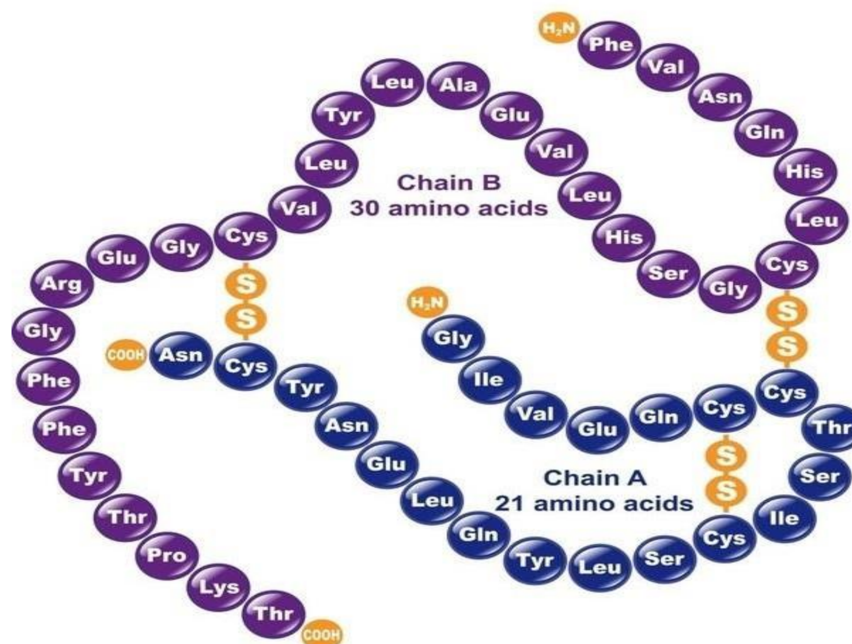


Fig. 1-3: Structure of two polypeptide chains sequence of insulin with total 51 amino acids (chain A 21 and chain B 30).

Also in A chain the residues 6 and 11 are linked by an intra-chain disulfide bridge. A and B chain are connected by C-chain, which is liberated along with insulin after the breakdown of proinsulin. The monomers of insulin molecule aggregate to form dimers and hexanes **(Ali *et al.*, 2017).** Insulin secretion from pancreatic β-cells is essential in humans and many other animals to maintain appropriate glucose

homoeostasis. Diabetes mellitus, a condition that has reached epidemic proportions across the world, is caused by faulty insulin production. Although β -cell breakdown is the cause of type 1 diabetes (T1DM), both decreased β-cell mass and loss of secretory function are implicated in type 2 diabetes (T2DM). Emerging evidence suggests that a functional defect, including the de-differentiation of the mature cells into a more progenitor-like state, may be a major cause of decreased secretion in T2DM (**Aloysius *et al.,* 2019).**

Beta-Cells are the most common cell type in the pancreatic islet and the only source of circulating insulin in animals.Scattered throughout the pancreas, accounting for around 1% of the total volume of the organ **(Mense and Rosol, 2018).** In rodents, β-cells form the core of the islet, with other endocrine cells, notably glucagon secreting α-cells and somatostatin-secreting δ-cells, along with smaller numbers of polypeptide P (PP) and ghrelin-expressing ε-cells, arranged towards the periphery **(Da Silva Xavier, 2018).** There is a broader mix of cell types in human islets, in this situation, the islet's ultimate compact shape is most likely the consequence of the folding of a trilaminar sheet composed of β-cells (outer layers) and cells (inner layers). The latter structure may allow for more contact between different cell types inside the islet, which might have physiological implications for insulin secretion control **(Rodriguez-Diaz, Molano *et al.,* 2018).** The most significant physiological secretagogue for insulin is glucose. Thus, the β-cell is ready to translate tiny variations in blood glucose concentration (usually 4.5 to 8 mM in humans) into substantial changes in insulin production within minutes.

Within β-cells, freshly manufactured insulin is first created as the prohormone proinsulin before being transformed into mature insulin by prohormone convertases (PC1, PC2, encoded by Pcsk1 and Pcsk2, respectively) **(Hussain, Harris *et al.,* 2018)** during trafficking through the secretory pathway. Active insulin is then stored in dense core secretory granules (5–10000 per cell) each having 300,000 or more insulin molecules Only a small percentage of the granules

are released by exocytosis (2% per hour at maximum glucose concentrations) is adequate to keep blood glucose levels within the above-mentioned physiological range. This strict control is necessary not just to prevent hyperglycemia, but also to prevent potentially fatal hypoglycemia caused by insulin over-secretion **(Xie, Zhu *et al.*, 2015).**

1.1.4.2. Biosynthesis of Mature Insulin

The beta islet cells of the pancreas help in the synthesis of insulin from the ultimate precursor molecule which is preproinsulin. The gene for this is located on chromosome 11 (**Tokarz *et al.*, 2018)** This inactive precursor molecule is released into cisternal space of rough endoplasmic reticulum where it is acted upon by proteolytic enzymes and is cleaved into proinsulin. This proinsulin molecule is then transported by micro vesicles to the Golgi apparatus, with a C (connecting) chain which links A and B chains. In the vesicles, proinsulin is released. In the maturing granules by the action of prohormone convertase 2 and 3 and carboxy peptidase H, the conversion of proinsulin to insulin continues **(Akai *et al.*, 2015)** With the help of microtubules and microfilaments, the translocation of the maturing granules occurs. During the secretion of mature granules into the circulation by the process of exocytosis **(Alim *et al.*, 2017)**, an equimolar ratio of insulin and C-peptide are released. In the islet cell secretion, about 6% composition is of proinsulin and zinc **(Wan *et al.*, 2017).**

1.1.4.3. Insulin Secretion

As a result of different stimuli like glucose, arginine and sulphonylureas, the beta cells of pancreas respond by secreting insulin **(Adam *et al.,* 2017).** Besides these stimuli, other factors which include neural, endocrine and pharmacological can also exert a stimulatory effect. The beta cells take glucose through GLUT-2 receptors **(Thorens, 2015).** During the entry of glucose into the beta cells, it is

oxidized by glucokinase, which acts as a glucose sensor. Glucose is phosphorylated to glucose-6- phosphate by the enzyme glucokinase generating ATP **(Fu *et al.*, 2013)**. If the levels of glucose are less than 90 mg/dl no secretion of insulin occurs that time. At such instances when the concentration of glucose is at sub stimulatory level, the efflux of K^+ through open K-ATP channels keeps the ß cell membrane at a negative potential at which voltage-gated Ca^{2+} channels are closed. However, the uptake and metabolism of glucose by the ß cell is enhanced as soon as plasma glucose levels rise **(Maimaiti *et al.*, 2017)** . Due to the increase in the concentration of ATP, membrane depolarization occurs and as a result voltage gated Ca^{2+} opens **(McTaggart *et al.*, 2010)** to the influx of Ca^{2+} the intracellular concentration of calcium increases and as a result of that exocytosis of insulin granules occurs **(Menting *et al.*, 2014).**

1.1.4.4. Physiological Role of Insulin

As a hormone insulin plays a significant role in regulating the supply of cellular energy, balancing macro nutrient and by directing the anabolic processes in the fed state **(Muniyappa and Sowers, 2013)**. In insulin-dependent tissues (muscle and adipose tissue) requires insulin for the intracellular transport of glucose in them. In muscle cells, insulin stimulates the synthesis of glycogen and lipid, while suppressing the process of lipolysis and gluconeogenesis. In the muscle cells when there is surplus supply of amino acids, at that time insulin is anabolic **(Piero *et al.*, 2015).**

Insulin is a hormone that is commonly linked to pancreatic secretion and blood sugar management. Long assumed to be "insulin-independent," research has revealed that insulin receptors (IR) are found on neurons, microglia, and astrocytes, among other cells. Insulin has a wide range of impacts on cells in the central nervous system, including both metabolic and non-metabolic processes. According to new research, insulin can promote neuronal survival or recovery following damage or during neurodegenerative disorders. Furthermore, research shows that

insulin has a substantial anti-inflammatory component, which may play a role in both neurotrauma and neurodegeneration. As a result, injection of exogenous insulin, either systemically or intranasal, is becoming a more important field of study in neurotrauma and neurodegenerative illnesses research **(Dyer, Vahdatpour *et al.*, 2016).**

Insulin entrance into the brain is strictly controlled by saturable insulin transporters on the blood-brain barrier (BBB). Insulin receptors (IR) are found in neurons and glia and are responsible for insulin transmission throughout the brain**,** the bulk of the insulin in the brain comes from the bloodstream. However, the capacity of neurons to manufacture insulin shows that insulin is required for appropriate function and development **(Stouffer, Woods *et al.,* 2015).** Insulin signaling is involved in the overall glucose metabolism of the brain and cerebral functions such as memory and cognition, insulin's cognitive-enhancing benefits in humans were initially observed in research utilizing systemic insulin infusions under euglycemic hyperinsulinemic circumstances **(Koury, Passos *et al*., 2013).**

1.1.4.5. Insulin Resistance and Insulin Sensitivity

Insulin resistance is frequently viewed as the key cellular abnormality in type 2 diabetes developments, finally, the combination of insulin resistance and consequent hyperinsulinemia, followed by hypoinsulinemia, results in hyperglycemia and the development of diabetes complications. Although insulin resistance develops prior to the beginning of overt illness, it has proven challenging to adequately diagnose in ordinary clinical practice. As a result of the critical clinical necessity of having precise diagnostic measures reflecting insulin resistance, decades of research in this field has been conducted in order to create new diagnostic tools **(Leslie, Palmer *et al.,* 2016)**. Insulin resistance is a critical aspect of type 2 diabetes and related disorders, and it is described as a condition in which more insulin is required to accomplish the same biological effects as a lower quantity of insulin in the normal state. As a result, while a simple oral glucose test

is frequently used to diagnose type 2 diabetes, it is not a strong indicator of insulin resistance. Clinical investigations, such as the hyperinsulinemic euglycemic clamp, which is considered as the "gold standard" for evaluating insulin resistance, or a modified insulin suppression test, can, of course, provide an accurate assessment of insulin resistance **(Broskey, Tam *et al.,* 2018).**

Insulin resistance in obesity and type 2 diabetes mellitus (T2DM) is caused by a complex interaction of numerous metabolic pathways, not just decreased insulin signaling. The examination of massive metabolomics and lipidomics data sets has provided fresh insight on the involvement of metabolites such as lipids, amino acids, and bile acids in altering insulin sensitivity. Metabolites can directly regulate insulin sensitivity by modulating insulin signaling pathway components such as insulin receptor substrates (IRSs) and AKT, and indirectly by altering substrate flux through multiple metabolic pathways such as lipogenesis, lipid oxidation, protein synthesis and degradation, and hepatic gluconeogenesis. Furthermore, post-translational protein modification by metabolites and lipids, such as acetylation and palmitoylation, can change protein function **(Yang, Vijayakumar *et al.,* 2018).**

1.1.4.5.1. Various Methods Applied for Insulin Resistance Determination

Insulin resistance contributes to the pathophysiology of diabetes and is a hallmark of obesity, metabolic syndrome, and many cardiovascular diseases. Therefore, quantifying insulin sensitivity/resistance in humans and animal models is of great importance for epidemiological studies, clinical and basic science investigations, and eventual use in clinical practice. Direct and indirect methods of varying complexity are currently employed for these purposes. Some methods rely on steady-state analysis of glucose and insulin, whereas others rely on dynamic testing. Each of these methods has distinct advantages and limitations. Thus, optimal choice and employment of a specific method depends on the nature of the studies being performed. Established direct methods for measuring insulin sensitivity in vivo are relatively complex. The hyperinsulinemic euglycemic

glucose clamp and the insulin suppression test directly assess insulin-mediated glucose utilization under steady-state conditions that are both labor and time intensive. A slightly less complex indirect method relies on minimal model analysis of a frequently sampled intravenous glucose tolerance test. Finally, simple surrogate indexes for insulin sensitivity/resistance are available (e.g., QUICKI, HOMA, 1/insulin, Matusda index) that are derived from blood insulin and glucose concentrations under fasting conditions (steady state) or after an oral glucose load (dynamic). In particular, the quantitative insulin sensitivity check index (QUICKI) has been validated extensively against the reference standard glucose clamp method. QUICKI is a simple, robust, accurate, reproducible method that appropriately predicts changes in insulin sensitivity after therapeutic interventions as well as the onset of diabetes. In this Frontiers article, we highlight merits, limitations, and appropriate use of current in vivo measures of insulin sensitivity/resistance (**Muniyappa and Madan 2018).**

Therefore, it is of great importance to develop tools for quantifying insulin sensitivity/resistance in humans and animal models that may be used to appropriately investigate the epidemiology, pathophysiological mechanisms, outcomes of therapeutic interventions, and clinical courses of patients with insulin resistance. Can be determine insulin resistance by many methods.

1.1.4.5.1.1. Direct Measurement of Insulin Sensitivity

A. Hyperinsulinemic Euglycemic Glucose Clamp

The glucose clamp technique is widely accepted as the reference standard for directly determining metabolic insulin sensitivity in humans. After an overnight fast, insulin is infused intravenously at a constant rate that may range from 5 to 120 $mU \cdot m^{-2} \cdot min^{-1}$ (dose per body surface area per minute). This constant insulin infusion results in a new steady-state insulin level that is above the fasting level (hyperinsulinemic) **(Muniyappa, Lee *et al.* 2018).** The main advantage of using the glucose clamp to estimate insulin sensitivity/resistance in humans is that it

directly measures whole body glucose disposal at a given level of insulinemia under steady-state conditions. Conceptually, the approach is straightforward, and there are a limited number of assumptions that are clearly defined. The main limitations of the glucose clamp approach are that it is time consuming, labor intensive, expensive, and requires an experienced operator to manage the technical difficulties.

B. Insulin Suppression Test

The insulin suppression test (IST), another method that directly measures metabolic insulin sensitivity/resistance, is intravenously infused to suppress endogenous secretion of insulin and glucagon. Simultaneously, insulin (25 $mU \cdot m^{-2} \cdot min^{-1}$) and glucose (240 $mg \cdot m^{-2} \cdot min^{-1}$) are infused into the same antecubital vein for 3 h. From the contralateral arm, blood samples for glucose and insulin determinations are taken every 30 min for 2.5 h and then at 10-min intervals from 150 to 180 min of the IST. The constant infusions of insulin and glucose will determine steady-state plasma insulin (SSPI) and glucose (SSPG) concentrations. The steady-state period is assumed to be from 150 to 180 min after initiation of the IST. SSPI concentrations are generally similar among subjects. Therefore, the SSPG concentration will be higher in insulin-resistant subjects and lower in insulin-sensitive subjects; i.e., SSPG values are inversely related to insulin sensitivity. The IST provides a direct measure (SSPG) of the ability of exogenous insulin to mediate disposal of an intravenous glucose load under steady-state conditions where endogenous insulin secretion is suppressed. **(Muniyappa, Lee *et al.* 2018).** The SSPG is a highly reproducible direct measurement of metabolic actions of insulin that is less labor intensive and less technically demanding than the glucose clamp. Indeed, since there are no variable infusions with the IST, steady-state conditions are more easily achieved with the IST than with the glucose clamp.

Many of the limitations of the IST are similar to those described above for the glucose clamp (with the exception that the IST is less technically demanding).

Thus, it is impractical to apply the IST in large epidemiological studies or in the clinical care setting. In exquisitely insulin-sensitive individuals, it is possible that subjects may become hypoglycemic during the IST. In individuals with type 2 diabetes, hyperglycemia may lead to glycosuria and underestimation of insulin resistance by SSPG (**Muniyappa, Lee *et al.* 2018**).

1.1.4.5.1.2. Simple Surrogate Indexes for Insulin Sensitivity / Resistance

A. Surrogates Derived from Fasting Steady-State Conditions

After an overnight fast, a single blood sample is taken for determination of blood glucose and plasma insulin. In healthy humans, the fasting condition represents a basal steady state where glucose is homo-statically maintained in the normal range such that insulin levels are not significantly changing and hepatic glucose production (**HGP**) is constant; i.e, basal insulin secretion by pancreatic β-cells determines a relatively constant level of insulinemia that will be lower or higher in accordance with insulin sensitivity/resistance such that HGP matches whole body glucose disposal under fasting conditions **(Broskey, Tam *et al.,* 2018).**

B. Surrogates Derived from Dynamic Tests

Surrogate indexes of insulin sensitivity that use information derived from dynamic tests such as oral glucose tolerance test (OGTT), meal tolerance tests, and intravenous GTT have been developed. Procedures for these tests have been described in Oral Glucose Tolerance Test/Meal Tolerance Test **(Cobelli, Toffolo *et al*. 2017).**

1.1.4.5.1.3. HOMA-IR Evaluation of Insulin Resistance

HOMA-IR evaluation of insulin resistance during perioperative period is the It uses only two values - your fasting insulin and glucose levels - and it counts not only your HOMA-IR parameter, but also your QUICKI index. These two values,

when put together, give you a better perspective on your health. Read the text to find out what is insulin resistance, if there are any insulin resistance symptoms, and how the HOMA formula calculation is done.

HOMA-IR homeostatic Model Assessment for Insulin Resistance - is an index used to determine if insulin resistance is present in a patient. People widely use it because of its simplicity and non invasive nature. In contrast, the 'gold standard' method for diagnosing insulin resistance (a hyperinsulinemic euglycemic metabolic clamp) involves multiple intravenous administrations of insulin and glucose, along with measuring blood parameters numerous times. Keep in mind, that this 'gold standard' method is limited to only scientific purposes. **(Muniyappa, Lee *et* al. 2018).**You can do your own HOMA formula calculation using one of the following equations. The choice depends on the units you prefer to use. Remember that both values refer to the fasting state - where nothing has been eaten or drank (other than water) for at least 8 hours before the blood sample is drawn**.**

HOMA-IR = (insulin x glucose) / 22.5 for the glucose concentration in mmol/l

HOMA-IR = (insulin x glucose) / 405 for glycemia in mg/dl. In both cases the insulin is in mU/l.

1.1.5. Chromium

1.1.5.1. Chemistry of Chromium Metal

Chromium is the most abundant mineral in Earth’s crust. Cr has an atomic number 24 in periodic table and has a relative atomic mass of 51.996 and it occurs in almost all oxidation states ranging from -2 to +6. But in environment Cr is mostly stable in trivalent and hexavalent form. Cr which is present in 0 oxidation state is biologically inert and is not naturally present in Earth’s crust while Cr (III) and Cr (VI) are originated from industries. The available form of chromium is as halides, oxides and sulphides. It is the +2 oxidation state of chromium which is unstable

and can be easily be oxidized to +3 forms in the presence of air **(Shekhawat *et al.*, 2015)**.

Chromium and its compounds gets absorbed in human body through the exposure to oral, dermal and inhalation routes. Cr (III) is less absorbed than Cr (VI) and this leads to a difference in their transport methods to cells. Cr (VI) enters into the cell via a non-specific anion channel by facilitated diffusion while Cr (III) enters by passive diffusion or phagocytosis. Human liver, kidney, spleen and bone have more concentration of Cr in comparison to other organs **(Chapin *et al.,* 2008).** Cr (VI) has the ability to easily penetrate in RBC. Because of its bioavailability Cr (VI) enters into RBC and gets converted into Cr (III) which binds to the cellular components and then it is unable to leave RBC. The structure of cells somewhat resembles to the structure of RBC due to this, Cr (VI) can be easily up taken by other cells. Also due to oral, intravenous and intra tracheal administration of Cr (VI) its level in tissues increases **(Shekhawat *et al.,* 2015)** absorption of Cr depends on some factors which are particle size, oxidation state and its solubility but majorly on the interaction with biomolecules in lungs. The main reduction of Cr (VI) to Cr (III) takes place in tissue of lungs.

1.1.5.2. Sources of Chromium Metals

The primary source of oral exposure to Cr for non-occupational human populations comes from food and drinking water. Cr levels in the food range from <10 to 1300 μg/kg, with the highest amount in meat, fish, fruits, and vegetables The concentration of Cr in uncontaminated water is very low, about 1–10 μg/L in rivers and lakes and 0.2–1 μg/L in rainwater, with an average concentration of 0.3 μg/L in ocean water **(Costa and Klein 2006).**

Increased industrial applications, however, lead to a large amount of Cr released into soil, ground water, and air. In 2009, the estimated releases of Cr compounds to surface water from domestic manufacturing and processing facilities were

486,063 lbs. The contamination of Cr(VI) in drinking water was first made known to the public in the Erin Brockovich (Film in 2000), depicting a southern California town of Hinkley. The elevated level of Cr(VI) in drinking water (usually several oob) has been reported in more than 30 US cities **(Sutton 2010)** posing an important question as to the health effect of Cr(VI) exposure in drinking water. The current drinking water standard established by the US Environmental Protection Agency (EPA) for total chromium is 0.1 mg/L or 100 ppb, However, there is no specific drinking water standard for hexavalent chromium. The California public health goal for Cr(VI) is 0.02 ppb which is a very low level and is often exceeded in public drinking water.

1.1.5.3. Roles of Chromium Metals

Due to the development of industry and economy, chromium (Cr) has been commonly used as well as released to the environment in a variety of industrial activities including electro plating, chromate manufacturing, leather tanning electro-planting, metal polishing etc. In contrast to organic contaminants, Cr is non-biodegradable and persistent in ecosystems. Cr exists mainly as Cr(VI) and Cr(III), while the former is much more soluble and mobile in aqueous solutions than the latter, which has caused extensive attention due to its carcinogenic, mutagenic and teratogenic effects on biological systems **(Agrafioti, Kalderis *et al.* 2014).** Therefore, it is imperative to remove Cr(VI) from wastewater prior to the discharge into water bodies.

All kinds of techniques have been applied to remove Cr(VI) from the aqueous solution, such as cyanide treatment, electro-chemical precipitation, reverse osmosis, adsorption, solvent extraction and ion exchange. Among these methods, adsorption is the most widely used because of its high efficiency and recovery of toxic and valuable metals from wastewater **(Xu, Xiao *et al.* 2011).** Some adsorbents like activated carbon, zeolite, iron oxide, fullerene, grapheme have been used for

Cr(VI) removal. However, these materials mentioned above have the defects of limited adsorption ability, aggregation or high cost.

Biochar is the porous carbonaceous by-product generated from biomass through pyrolysis/carbonization under anoxic and anaerobic conditions. When applied to soils, biochar can not only increase soil fertility, raise agriculture productivity and enhance soil water holding capacity **(Janus, Pelfrêne *et al.* 2015)** but also serve as carbon storage to reduce CO_2 emissions and mitigate climate change.Apart from soil application, recent studies have focused on biochar's potential ability on removing various contaminants (heavy metal, organic pollutants and microbial contaminants) from wastewater system, due to its accessible and abundant in feedstock materials.

1.1.5.4. General Pathways for Chromium Exposure

Chromium is ubiquitous in nature and ranges in different concentration in environment. Cr (III) is usually present in environment and Cr (VI) is totally produced by human activities. General exposure to Cr (VI) is through emissions from industries. Cr (VI) is used as anticorrosive agent in various cooling system, combustion eg- Cigarette smoke and ash from power plants **(OEHHA, 2000)**. Mostly, all food materials contain some amount of Cr ranging from 20-500µg/Kg. The highest level of Cr in food founds in meats, mollusks and crustaceans (U.S. EPA, 1985). A worker who works in Cr industries experiences the highest exposures to Cr(VI) and mainly the exposure is through respiratory and dermal routes **(Chapin *et al.*, 2008)**. The major source for chromium exposure is food. The oral intake for infants of 1 yr is 33-45 µg/day, for children of 11 yr is 123-171 µg/day and for adults it is 246-343 µg/day **(Wu *et al.*, 2000)**.

The most common exposures routes for chromium are as under

- **a.** Ingestion
- **b.** Dermal contact

c. Inhalation

Human health is adversely affected due to the exposure of chromium and these health effects are categorized in two types, carcinogenic and non-carcinogenic and have three types of exposure duration(**Sarbassov *et al.*, 2004**):

1. Acute (14 days or less)
2. Intermediate (75-364 days)
3. Chronic (365 days or more)

But Unites States Environmental Protection Agency had reported that chromium is carcinogenic only if taken.

1.1.5.5. Mechanism of Cr(III) and Insulin Action

The mechanism of Cr(III) action at a molecular level has proven elusive. Researchers can generally agree that Cr(III) enhances the uptake of glucose by cultured or freshly isolated adipocytes or skeletal muscle cells in an insulin-dependent manner. Increased movement of GLUT4 to the plasma membrane appears to be responsible for the increased glucose uptake (**Doerner III *et al.*, 2014**). For example, observed rat skeletal muscle perfused with a solution containing CrCl3 and insulin had a greater increase of glucose uptake than tissue treated only with insulin. The tissue also had greater levels of plasma membrane associated GLUT4 after treatment with Cr(III) and insulin opposed to insulin alone. Two members of the insulin-signaling cascade were also examined. No effects were observed on Akt threonine-308 or AS160 (a substrate of Akt) threonine-642 phosphorylation for the CrCl3 and insulin-treated group compared with the insulin-only group.

In addition to glucose transport and GLUT4 movement, the consensus stops. Most research has focused on the insulin signaling pathway for the site of Cr(III) action. Several elements of the insulin signal pathway have been implicated as the

site of Cr(III) action; however, contradictory results between laboratories have resulted. Whether this is a problem with using different organisms, tissues, or cell types; different Cr(III) compounds; other varying experimental conditions; or other factors is unclear. As an example, skeletal muscle from Holstein calves that had received Cr(III) as Cr-enriched yeast perorally for 70 days possessed greater levels of IRS-1 phosphorylated at tyrosine-632 (but not serine-307) and Akt phosphorylated at serine-473 **(Jovanović *et al.*, 2017)**.

The problems with conflicting results can be observed by comparing the results of these two studies on skeletal muscle. The first is inconsistent with an insulin-signaling pathway-dependent increase in GLUT4 migration to the plasma membrane whereas the latter is consistent with an effect from Cr very early in the insulin-signaling pathway at insulin receptor or at IRS-1. Some recent articles have suggested Cr(III) supplementation results in increases in mRNA levels for members of the insulin-signaling cascade including insulin receptor, IRS-1 or IRS-2, PI3K (phosphatidylinositol-3-kinase), and Akt (protein kinase B) with concomitant reductions in mRNA levels for glycogen synthase kinase-3 in skeletal muscle and/or liver in animals from fish to rats and mice to pigs **(Cui *et al.*, 2019)**. However, these results are not always consistent and are not always accompanied by the corresponding changes in protein concentrations. Attention has also recently been focused on AMP-activated protein kinase (AMPK) as a site of Cr(III) action independent of the insulin-signaling pathway **(Nair, 2019)**. Cr(III) supplementation has been suggested to also increase mRNA levels of subunits of AMPK9, for example **(Liu *et al.,* 2017).**

Aim of the Study:

The presented work aimed:

1. To investigate a genetic mutation (3243) of mitochondrial DNA in Iraqi type 2 diabetic patients with/without ischemic heart diseases of Kerbala province.

2. Study the chromium level of chromium ion in type 2 diabetes mellitus with/without ischemic heart diseases.
3. Flow up of others biomarker such as lipid profile, fasting blood sugar, insulin and HbA1c % and link them with chromium ion level and suggest a solution of increase chromium ion level when patients with chromium deficiency

CHAPTER TWO

Materials and Methods

2. Materials and Methods

2.1. Patients

The current study was cross-sectional study. Two study groups were selected, first group for the presented by diabetes mellitus type 2 with ischemic heart disease (50 patient), and the second group of the presented by type 2 diabetes mellitus without ischemic heart disease (50 patients). The study was managed throughout the period since from first Nov., 2020 to Aug., 2021. The sample collected from Kerbala heart center, Al-Hassan Center for Endocrinology in Al-Hussein Teaching Hospital, Al-Hussein Medical City, Kerbala Health Directorates / Kerbala – Iraq. The parameters investigations and molecular studies were done in the laboratories of Department of Chemistry and Biochemistry, College of Medicine, University of Kerbala and Al-Hussein Teaching Hospital laboratories' of AL-Imam AL-Hussein medical city.

2.1.1. Approval of the Ethical Committee

The protocols of the study were approved by Ethical Committee, College of Medicine, University of Kerbala after a verbal written informed consent for participation and for taking a blood and urine samples for investigations from everyone enrolled in this study.

2.1.2. Blood sampling

Five milliliters of blood was drawn by vein puncture from all individuals participated in this study after taking the patient's consent. The collected blood was divided into three parts:

1. One ml of blood that used for gene analysis, collected in EDTA containing tube and used for DNA extraction, then were analyzed directly to obtain high purity of DNA.

2. One ml placed in EDTA containing tube for analyzing HbA1c test.
3. Three ml of blood placed in gel tube. It was left fifteen minutes at room temperature for coagulation. Blood was centrifuged for 15 minutes at 3000 x g. Serum was collected, then frozen till analyses for measuring the biomarker and chromium ion. The samples were put it in package containing ice for frozen samples after collected and transfer it to the laboratory.

2.2. Materials

2.2.1. Chemicals

Various chemicals and kits were purchased and used in various stages of the current study are presented in table (2.1).

Table 2.1: The kits and chemicals used in this study

Chemicals	Source (country)
mitDNA extraction kit	Cyntol (Russia)
Master Mix kit (Go taq hot start green)	Cyntol (Russia)
Nuclease free water	Cyntol (Russia)
Primers Cyntol company	Cyntol (Russia)
100 - 1000 bp DNA leader	Cyntol (Russia)
Ethidium bromide	Promega (U.S.A)
Agarose analytical grade	Promega (U.S.A)
Tris borate EDTA (TBE) Buffer X10	Promega (U.S.A)
Cholesterol kit	Roche (Germany)
Triglyceride kit	Roche (Germany)
LDL kit	Roche (Germany)
HDL kit	Roche (Germany)
Glucose Kit	Roche (Germany)
HbA1c Kit	Roche (Germany)

Insulin Kit	Roche (Germany)
Chromium stander solution	Certipur (Germany)

2.2.2. Apparatus and Equipment's

Apparatus and instruments used in various methods of the current study are presented in table (2.2).

Table 2.2: Instruments and Apparatus

Apparatus	Source
Autoclave	Hirayama (Germany)
Bench centrifuge	Hettichi (Germany)
Water stiller	England
Gel electrophoresis system	Biometra (Germany)
Hood	C.B.S scientific (USA)
Magnetic stirrer	Japan
Minispin centrifuge	Eppendorf (Germany)
Nano drop	Bio drop (England)
Oven	Binder (Germany)
PCR-thermocycler	Biometra (Germany)
Photo documentation	UVP (UK)
Rotater (Rotisserie mixer)	Greiner Laborgerate (England)
Sensitive balance	Sartorius (Germany)
UV source	USA
Vortex mixer	Cyan (Belgium)
Water bath	Memmert (Germany)
COBAS INTEGRA-400 plus	Roche (Germany)
COBAS E-411	Roche (Germany)

Atomic Absorption Spectrophotometry AA-6300	SHIMADZU (Japan)

2.3. Methods

2.3.1. Determination of Body Mass Index

The body mass index (BMI) was estimated by this equation:

$$\textbf{BMI, kg/m}^2 = \textbf{Weigh (kg) / Hight (m}^2\textbf{)}$$

For both patients groups, weight condition was classified according to their BMI as shown below **(World Health Organization, 2016).**

Weight status	BMI (kg/m^2)
Under weight	< 18.5 kg/m^2
Normal weight	18.5 to 24.9 kg/m^2
Over weight	25.0 to 29.9 kg/m^2
Obese	≥ 30.0 kg/m^2

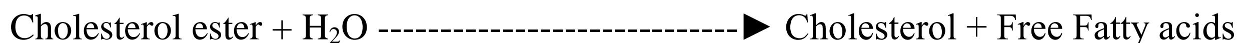
2.3.2. Determination of Serum Lipid Profile

2.3.2.1. Determination of Serum Total Cholesterol Level

Principle

Determination of total cholesterol concentration is performed by using three enzymes: cholesterol esterase (CE), cholesterol oxidase (CO) and peroxidase (POD) which based on the production of colored complex. The intensity of the color generated is proportional to the concentration of cholesterol in the sample which determined by estimate the absorbance at 500 nm **(Friedewald *et al.*, 1972)** as shown in the following reactions:

Cholesterol esterase



Cholesterol ester + H_2O ----------------------------► Cholesterol + Free Fatty acids

Cholesterol oxidase

Cholesterol + O_2 --------------------------------► Cholesterol-4-en-one 3 + H_2O_2

Peroxidase

$2H_2O_2$ + phenol + 4-amino-antipyrine --------------►Quinonimine (pink) + $4H_2O$

Reagents:

Table 2.3: Reagents for determination of Total Cholesterol Concentration

	Contents	**Concentrations**
Reagent 1	PIPES buffer	200 mmol / L, pH 7.0
	Sodium cholate	1mmol / L
	4-aminophenazone	0.33 mmol / L
	Phenol	4 mmol / L
	Cholesterol esterase	>250 U / L
	Cholesterol oxidase	> 250 U / L
	Peroxidase	> KU / L

Reference values : Adult: <200 mg/dL (<5.18 mmol / L)

<18 years: <170 mg/dL

Calculation: Automatic calculation by using the Roche COBAS c311.

2.3.2.2. Determination of High Density Lipoprotein - Cholesterol

Principle

This technique1 based on "precipitation method " involve firstly using of selective precipitation regents such as (heparin and dextran sulphate or

phosphotungstic acid with magnesium ions Mg^{+2} as $MgCl_2$) which separate apoliprotein B-containing lipoproteins (VLDL and LDL), after centrifuge "HDL-C" obtained in the supernatant treated as total cholesterol, following the same steps which use in determination of total cholesterol, **(Tietz, 1999).**

Reagents

Table 2.4 : Reagents for determination of HDL-Cholesterol

	Content	**Concentration**
Reagent 1	PIPES buffer	200 mmol / L, pH 7.0
	Sodium Cholate	1.0 mmol / L
	4-Aminophenazone	0.33 mmol / L
	Phenol	4 mmol / L
	Cholesterol esterase	>250 U / L
	Cholesterol oxidase	> 250 U / L
	Peroxidase	> KU / L

Reference values: Adult: <200 mg/dL (<5.18 mmol / L)

<18 years: <170 mg/dL

Calculation: Automatic calculation by using the Roche COBAS c311.

2.3.2.3. Determination of Triglyceride Concentration

Principle

Determination of TG in serum involve the enzymatic splitting of TG to glycerol and free fatty acids (FFA) by lipase enzyme Then the glycerol is phosphorylated by adenosin triphosphate (ATP) in the presence of glycerolkinase (GK) to form

glycerol-3-phosphate (G-3-P) and adenosinediphosphate (ADP). G-3-P is oxidized by glycerophosphate oxidase (GPO) to produce dihydroxyacetone phosphate (DHAP) and hydrogen peroxide, after that peroxidase enzyme (POD) catalyze the production of red color complex by coupling of 4-aminoantipyrine (4-AA) and phenol with hydrogenperoxide (H_2O_2). The increase in absorbance is directly proportional to the concentration of TG in the serum **(Lafta, 2014).**

Lipase

Triglycerides -----------------------► Glycerol + Free fatty acids

Glycerol Kinase

Glycerol + ATP -----------------------------► Glycerol-3-phosphate + ADP

GPO

Glycerol-3-phosphate + O_2 -------------► Dihydroxyacetonephosphate + H_2O_2

Peroxidase

H_2O_2 + 4-Chlorophenol + 4-AA --------------------► Quinoneimine + H_2O

Reagents

Table 2.4 : Reagents for determination of triglycerides Concentration

	Contents	Concentrations
Reagent	PIPES buffer	50 mmol/L, pH 6.8
	Mg^{2+}	40 mmol/L
	Sodium cholate	0.20 mmol/L
	ATP	>1.4 mmol/L
	4-Aminophenazone	>0.13 mmol/L
	4-Chlorophenol	4.7 mmol/L
	Lipoprotein lipase	>83 ukat/L
	Glycerokinase	>3 ukat/L
	Glycerol phosphate oxidase	>41 ukat/L
	Peroxidase	>1.6 ukat/L

Reference Values

• Normal: 150 mg/dL (1.70 mmol/L)

• Borderline/high : 150-199 mg/dL (1.70-2.25 mmol/L)

• High: 200-499 mg/dL (2.26-5.63 mmol/L)

Calculation: Automatic calculation by using the Roche COBAS c311.

2.3.2.4. Determination of Low Density Lipoprotein - Cholesterol

Principle

Direct determination of serum low-density lipoprotein-cholesterol (LDL-C) based on the specific precipitation of LDL-C by polyvinyl sulfate in serum and subsequent test as residual cholesterol of the rest of lipoproteins (VLDL+ HDL) remaining in the clear supernatant. LDL-C obtained in the supernatant treated as total cholesterol following the same steps which use in estimation total cholesterol **(Tietz, 1999)**

Refrence Values: less than 150 mg / dl

2.3.2.4. Estimation of Very Low Density Lipoprotein

Serum VLDL-cholesterol level can be calculated by using specific equation: **(Friedewald et al., 1972)**

VLDL-Cholesterol = Triglyceride / 5 ; When TG given in mg/dL

2.3.3. Determination of Serum Glucose Level

Principle

Serum glucose concentration was determined by using coupled enzymatic reaction in which hexokinase enzyme (HK) catalyzes the phosphorylation of glucose by ATP to form glucose-6-phosphate and ADP. To follow the reaction, a second

enzyme, glucose-6-phosphate dehydrogenase (G6PDH) is used to catalyze oxidation of glucose-6-phosphate by $NADP^+$ to form NADPH + H^+. The concentration of the NADPH + H^+ formed is directly proportional to the glucose concentration. It is determined by measuring the increase in absorbance at 340 nm **(Wu *et al.*, 2006).**

Hexokinase

G6PDH

Reagents

	Content	Concentration
Reagent 1	MES buffer	5 mmol/L, pH 6
	Mg^{+2}	24 mmol/ L
	ATP	4.5 mmol /L
	$NADP^+$	7 mmol/L
Reagent 2	Hexokinase	300 UKat / L
	Glucose-6- Phosphate dehydrogenase	300 UKat / L

Reference Values

Normal fasting blood sugar level less than 100 (mg/dl)

A fasting blood sugar level (100 -1256mg/dl) is considered pre diabetes.

A fasting blood sugar more than 126 (mg/dl) is considered diabetes **(Ketema and Kibret, 2015).**

Calculation : Automatic calculation by using the Roche COBAS c311.

2.3.4. Determination of HbA1c%

Determination of glycated hemoglobin (HbA1c) by "COBAS INTEGRA© 400 plus " with "COBAS HbA1c kit" , the normal level less than 7% but risk level equal or more than 7%.

Principle

This method involve firstly addition of tetradecyltrimethylamonium bromide (TTAB) on the whole blood as the hemolyzing reagent to eliminate interference from leukocytes (TTAB does not lyse leukocytes), the second step involve addition of R1 (antibody reagent), glycohemoglobin (HbA1c) which glycated at N-terminal of B-chain in the sample react with (anti-HbA1c AB) and cause agglutination. The agglutination rate is photometrically determined at 550 nm and related to the HbA1c concentration. The final result is calculated automatically expressed as a percentageof HbA1c **(Metus *et al.*, 1999).**

2.3.5. Determination of serum Insulin Concentration

The ARCHITECT Insulin assay is used for the quantitative determination of human Insulin in human serum on the ARCHITECT PLUS i 1000 SR.

Principle

The ARCHITECT Insulin assay is one-step immunoassay to determine the presence of human insulin in human serum or plasma with flexible assay protocols.

1. Sample, anti-insulin coated paramagnetic microparticles and anti-insulin acridinium-labeled conjugate are combined to create a reaction mixture. The Insulin present the sample to the anti-insulin coated microparticles and anti-insulin acridinium labeled conjugate.
2. After washing, pre-trigger and trigger solutions are then added to the reaction mixture.

3. The resulting reaction is measured as relative light units (rlus). There is a direct relationship between the amount of insulin in the sample and the ruls detected by the ARCHITECT i System optics.

Calculations: Automatic calculation by using the ARCHITECT PLUS i 1000 SR.

2.3.6. Estimation of Insulin Resistance

HOMA-IR stands for Homeostatic Model Assessment of Insulin Resistance. It marks for both the presence and extent of any insulin resistance that might currently express. It is a terrific way to reveal the dynamic between baseline (fasting) blood sugar and the responsive hormone insulin. The HOMA-IR is an approximating equation for insulin resistance **(Matthews et al., 1985)**. HOMA-IR was calculated using the formula:

HOMA-IR = Fasting insulin, (mlU/L) x Fasting glucose, (mg/dL) / 405

Healthy Range: **(0.5–1.4)**

Less than 1.0 means insulin-sensitive which is optimal. Above 1.9 indicates early insulin resistance. Above 2.9 indicates significant insulin resistance.

2.3.7. Determination of chromium ion

Estimation of chromium ion by SHIMADZU AA – 6300 (Atomic absoption spectrophotometry) with stander solution of chroium ion (1000 mg/l).

Principle

The process of atomic absorption involves the absorption of radiant energy at specific wavelengths by the elements in a gaseous state.

$$\text{Absorption} = -\log(I_t / I_0)$$

The process of atomic emission involves the release of radiant energy at specific wavelengths by the elements in a gaseous state. The absorption of the radiant energy is not a nuclear process, but involves the valence electrons where the absorbed radiant energy elevates an electron to the next higher orbital energy level.
Each element will absorb at a wavelength that is particular to that element, and then release the energy at a wavelength that is also specific to that element. These two processes, atomic absorption and atomic emission, are utilized for elemental analysis.

Another approach to elemental analysis is electrothermal atomization using graphite furnaces. In this approach, a small sample volume (3–5 μl) is deposited onto a platform in the center of a small graphite tube. The tube is then electrically heated, which in turn atomizes the sample. The lamp is aligned to pass through the center of the tube for the atomic absorption. Figure 2.1(a) depicts examples of graphite tubes used in the graphite furnace.

Note the hole in the middle of the tube. This is where the sample is introduced. Figure 2.1(b) depicts a graphite tube with a platform for sample addition. Figure 2.2. shows a graphite furnace sample introduction source where a robotic arm with a sample tube will insert the tube in the middle of the graphite tube and deposit the sample. Once the sample is introduced into the graphite tube there are three stages that the graphite tube goes through.

First, the temperature of the tube is slightly elevated for the sample drying stage at 125 °C for 20 sec. Second, the temperature is increased for ashing of the organic matter present. For sodium (Na), the ashing temperature is 900 °C for 60 sec. Molecular species have broad absorption bands and will interfere with the narrow absorption bands of the elements being measured. Ashing will help ensure they are removed and will not interfere. Third, the temperature is raised further for vaporization of the analyte sodium atoms at 1500 °C for 10 sec.

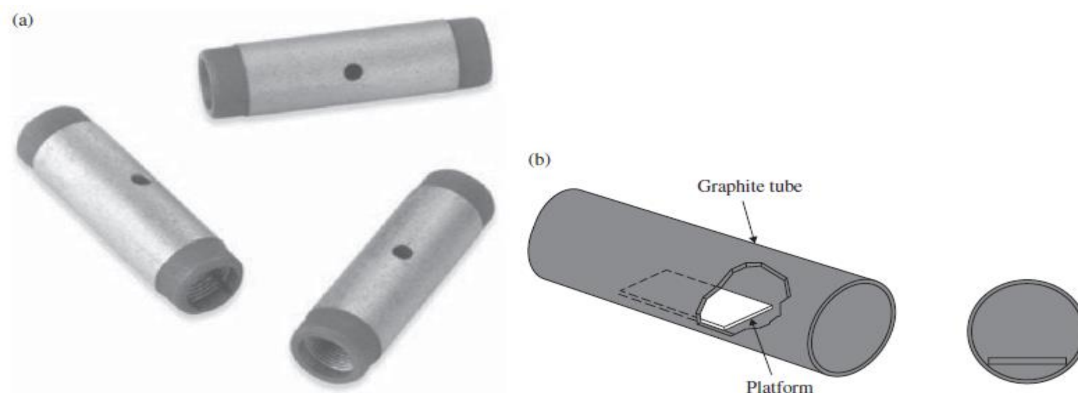


Fig: 2.1 (a) Examples of graphite tubes used in the graphite furnace including a hole for sample introduction. (b) Illustration of the sample platform that is inside of the graphite tube.

Fig: 2.2 Graphite furnace sample introduction system. The robotic arm holds a sample introduction tube that is inserted in the middle hole of t graphite tube where the sample is introduced.

The two main gas mixtures used for the source flames include air– acetylene and nitrous oxide–acetylene.The air–acetylene is used for elements that are not prone to refractory conditions. Refractory conditions exist where the element exists as an oxide that is not converted to the gaseous element in the flame **(Elwell and Gidley, 2013).**

Calculations

Automatic calculation by using SHIMADZU AA – 6300 (Atomic absoption spectrophotometry)

Reagent:

Prepare 5 stander soluitions from stock solution 1000 ppm, 2.5, 5, 7.5, 10 and 12.5 ppb, Figure 2.4.

wave length = 357.9 nm

peak = 357.7 nm

Lamp current low = 10 (mA)

Lamp mode = BGC-D2

Slit width = 0.7 nm

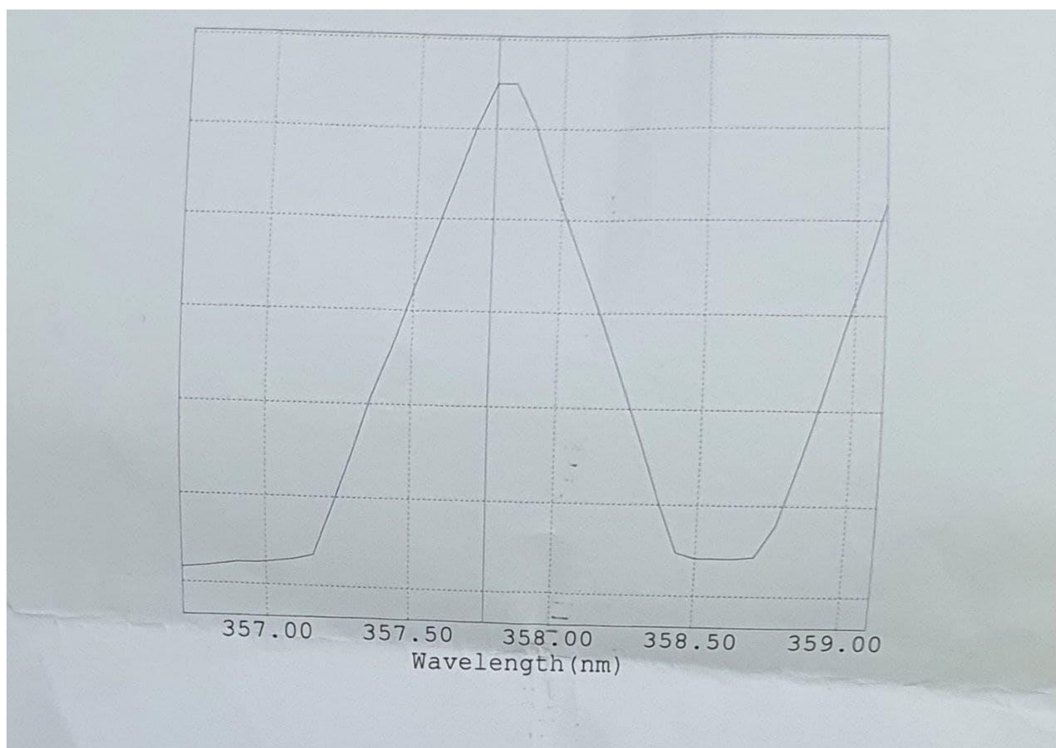


Fig. 2.3: Determination of peak wave length

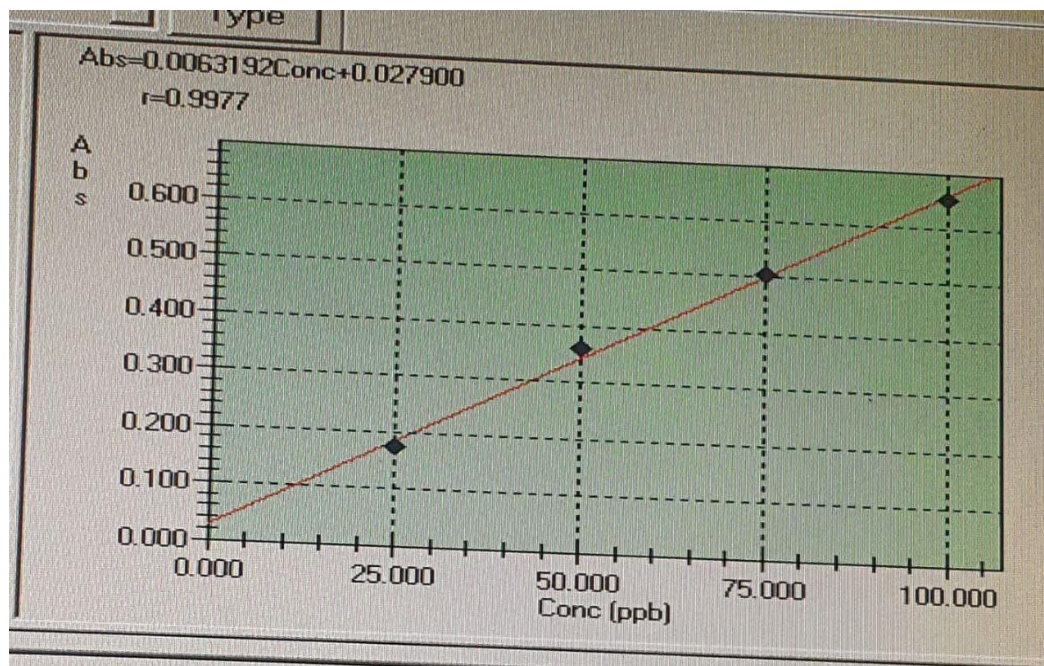


Fig. 2.4: Determination of concetration of stander solutions

***Refrence Values**

0.08 mcg/L – 0.5 mcg/L equal (8 ppb – 50 ppb).

2.4. Molecular Analysis

Mitochondrial DNA (mitDNA) will be extracted from blood using DNA isolation kit (cyntol) genotyping will be carried out by allele-specific ARMS-PCR for –3243A/G SNP of PRKCB1 (A3243G), primers and a master mix kit (Promega) were used, PCR products were separated on a 1.5% agarose gel electrophoresis.

2.4. 1. DNA Extraction

MitDNA was extracted from whole blood that collected from patient and control groups by using"G-spinTM Total DNA Extraction Kit" (cyntol).

Principle

Nucleic acids isolation principle of the “RealBest extraction 100” kit is in temperature treating of samples by multicomponent lysis reagent that destructs the nucleoprotein complex, followed by precipitation of nucleic acids onto magnetic

particles with alcohol, wash procedure and subsequent elution. The sample is then ready for PCR or RT-PCR test run.

The principle of the assay is based on the extraction of nucleic acids from samples with preliminarily added internal control sample (IC) and running the reaction of reverse transcription and amplification of selected RNA fragments or amplification of selected DNA fragment with detection of PCR products in real time.

For the convenience of working with a kit a use of a magnetic stand is recommended. Nucleic acids can be extracted from 100 µl and 200 µl of the sample.

The PCR kits of the "RealBest" series include a positive control (PC) sample corresponding to a given PCR assay kit. PC must undergo the isolation procedure together with biological samples and negative control (NC) sample.

Procedure

1. To prepare the suspension add 250 µl of SSP (solution for sample preparation) to the tubes with ticks. Place the tubes into homogenizer and perform the grinding procedure.
2. Add 30 µl of SSP to the tubes with ticks (in the case of analysis of full or large ticks add 50 µl of SSP). Place the tubes into a chilled to minus (20-30 °C) stand-fridge and keep for at least 20 minutes in the freezer at minus (20-30 °C). Next, place the rack with the analyzed samples into a container with ice or refrigerant. Take a tube with the tick frozen in the SSP, and as quickly as possible, without waiting for the thawing of the solution, thoroughly crush the tick with separate sterile pestle. Without removing the pestle, put a tube with crushed tick into the rack, placed in ice. Add **200 µl** of chilled SSP to the tube. Gently rinse the pestle in the tube, pull it out and put in a disinfectant solution. Stir the contents of the tube on a shaker (5-10 seconds). Perform the grinding procedure with other samples. Collect the contents from the walls of the tube with brief centrifugation. Transportation and storage of ticks and tick suspension samples, repeated freezing and thawing is not allowed.

- up to 24 hours at 2-8 °C
- up to 2 weeks at minus (18-60) °C.

Extraction Protocol:

1. Determine the appropriate number of reaction tubes needed for patient specimen and control testing. Label each 2.0 ml tube for each patient specimen and control sample. Add 30 µl of IC solution to each tube, then add 100 µl of negative control to the tube marked "NC". After that add 70 µl of Negative Control and 30 µl of positive control to the tube marked "PC". Then Add 100 µl of each specimen to the appropriately labelled tube. For higher sensitivity add 50 µl of specimens.
2. Add 300 µl or lysis reagent with sorbent to each tube. Vortex for 10-15 seconds. Place the tubes into thermo shaker, and incubate for 10 minutes at 65 °C and 1300 rpm. Spin shortly to collect the drops. In case the specimen is not lysed completely transfer the contents to the other tube careful not to touch the pellet.
3. Add 400 µl of solution for DNA/RNA precipitation in each tube. Vortex for 10-15 seconds. Centrifuge at 13000 rpm for 5 minutes at temperature 18-25 ºC.
4. Trying not to shake up a pellet, place the tubes into magnetic stand. Using a new tip for each sample, carefully remove the supernatant without disturbing the pellet. Add 500 µl of Wash Solution № 1 in each tube. Vortex vigorously for 10-15 seconds. Centrifuge at 13000 rpm for 2 minutes.
5. Trying not to shake up a pellet, place the tubes into Magnetic Stand. Using a new tip for each sample, carefully remove the supernatant without disturbing the pellet.
6. Add 300 µl or Wash Solution № 2 to each tube. Vortex vigorously for 10-15 seconds. Centrifuge at 13000 rpm for 2 minutes.

7. Trying not to shake up a pellet, place the tubes to Magnetic Stand. Using a new tip for each sample, carefully remove the supernatant without disturbing the pellet.
8. Dry the pellet with open caps for 2-3 minutes at room temperature (18-25) °C.
9. Add 200 µl of Specimen Diluent to the tube if the number of assays performed with this probe will account to 1-3.
10. Add 600 µl of Specimen Diluent to the tube if the number of assays performed with this probe is higher. Vortex vigorously for 10-15 seconds. Place the tubes into Thermo Shaker, and incubate for 5 minutes at 65°C and 1300 rpm. Then centrifuge for 1 minute at 13000 rpm. Samples are ready for PCR or reverse transcription and PCR.

2.4.2. Polymerase Chain Reaction

Principle

Polymerase Chain Reaction (PCR) is a technique used for amplify a small amount of template DNA into billion copies in a few hours. The reaction done by mixing the DNA with primers (small segment of DNA essential for DNA polymerase enzyme and complementary to template DNA) on either side of the DNA (forward and reverse), free nucleotides (dNTPs for DNA, NTPs for RNA), Taq polymerase (enzyme extracted from the bacterium Thermus aquaticus, that normally live at high temperature) and buffer. The temperature is then shift between hot and cold in order to denature and re-anneal the DNA, with the polymerase adding new complementary strands each time(Garibyan and Avashia, 2013). PCR reaction cycle for 30 times and involve 3 major steps :

1. **Denaturation**: This stage done at 95 °C involve separate the double strand of DNA into 2 single strand .
2. **Annealing**: This step done at 55-65 °C. at this stage the primers anneal to the ends of the template strands of the DNA and begin the reaction.

3. **Extension**: This step occurs at 72-74 °C and involve the extension of the primers to form a new strand that is complementary to the template strand.

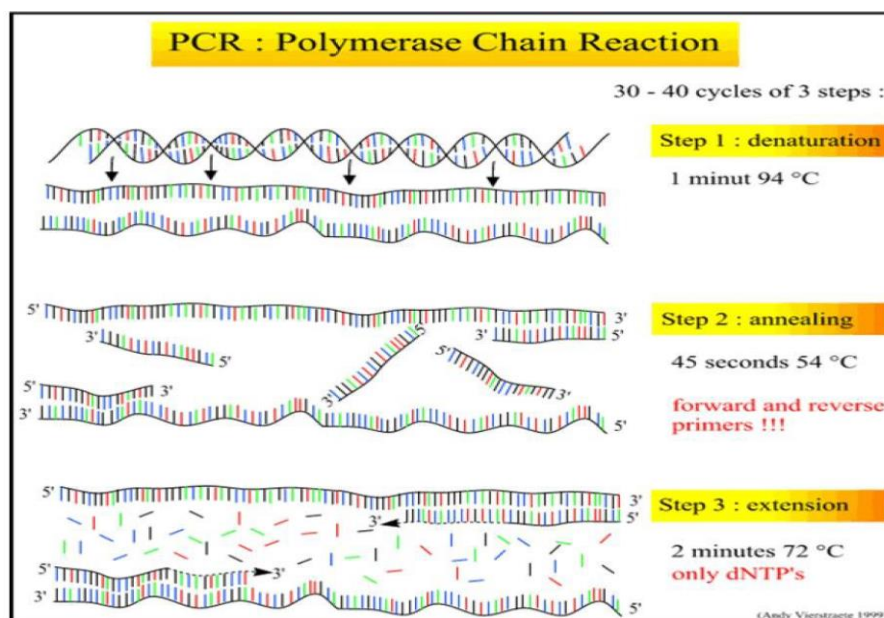


Fig. 2.4: Principle of Basic of PCR stages

2.4.2.1. Primers Designing

In our study we used national center for biotechnology information NCBI to design sequences and mutation. A triprimer allele specific ARMS-PCR designing for the detection of –3243 A/G polymorphism of MTLL1 as shown in the table (2.6).

Table 2.6: Primer sequence for alleles of A3243G

Primers	Sequence
Mutant forward	5´…GCA AGA GAT ACA GTG TTG CTC CA…3´
Common Reverse	3´…CGT TCT CTA TGT CAC AAC GAG GT…5´

2.4.2.2. Amplification Refractory Mutation System

The amplification-refractory mutation system (ARMS), which is also termed as "allele-specific polymerase chain reaction (ASPCR) or PCR amplification of specific alleles", is considered a simple, fast , and reliable technique for detecting any mutation include single base changes. ARMS is based on the use of sequence-specific PCR primers that promote amplification of test DNA only when the target

allele is included within the specimen andwill not amplify the nontarget allele. Following an ARMS reaction the existence or absenceof a PCR product is detection for the existence or absence of the target allele.

For the mutant-specific primer (M), the 3′ terminal base of the ARMS primer should be complementary to the mutation sequence ; for the normal-specific primer (N), the 3′ terminal base should be complementary to the corresponding normal sequence **(Little, 1995).**

2.4.2.3. Primers

A primer is a short strand of DNA which act as a starting point for DNA replication. It is essential for DNA replication becauseDNA polymerase {enzyme catalyze replication process} can only add new nucleotides to an existing strand of DNA. This enzyme starts replication at the 3'-end of the primer, and copies the opposite strand. Primers were constructed by (primers cyntol company) in lyophilized state after that The subsequent steps involve the reconstitution and dilution of the primers:

1. The tube was spin down before opening the cap.
2. The dilution of primers as follow:
   - Normal Forward Primer : 690 µl of Nuclease free water
   - Mutant Forward Primer : 460 µl of Nuclease free water
   - Common Reverse Primer : 662 µl of Nuclease free water
3. Each primer after the first dilution well mixed by suitable vortex to obtain { stock solution}.
4. 10 µl of the stock solution were transported to a 0.5 ml eppendorf tube and added 90 µl of nuclease free water to obtain {working solution}. Both stock and working solutions were kept at -20 ^{o}C.

2.4.2.4. Optimization of PCR Assay

Green Master Mix is a (ready-to-use solution)encompass bacterially derived TaqDNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR.

The condition that show best result was selected, as shown in (table 2.6), for a 20 µl reaction volume the volumes were added in a PCR tube and centrifuged at 2000 xg for 30 seconds in a micro centrifuge for mixing then the samples placed in thermo cycle.

Reagent	Volume
Forward primer	2µl
Reverse Primer	2µl
Master Mix	10µl
Nuclease Free Water	5µl
DNA template	5µl
Total Volume	25µl

2.4.3.5. Thermocycler Program for DNA Amplification

The PCR reaction program procedures for SNP (-1504 C / T) in PRKCB-1 was presented in table (2.7).

Table 2.9: PCR reaction program

Type of Cycle	Temperature °C	Time	No. of Cycle
Intial denaturation	**95**	**5 min**	**1 cycle**
Denaturation	**95**	**30 sec**	
Anealing	**61**	**30 sec**	**35 cycle**
Extension	**72**	**60 sec**	
Final Extension	**72**	**5 min**	
Total Time : 1 hour and 35 min			

2.5. Agarose Gel Electrophoresis

2.5.1. Agarose Gel Prepration

Fifty ml of 1% agarose solution was prepared according to the following steps:

A. **Preparation of Solution:** 1X TBE buffer (tris borate EDTA) was prepared by diluting 10X TBE buffer with deionized water (10 ml of 10X TBE buffer with 90 ml of deionized water: 1:10 dilution.

B. **Preparation of Agarose Gel**

1. Firstly 0.5 gm of agarose were weighted and placed into a conical flask, and then 50 ml of 1X (TBE) buffer was added and mixed gently.
2. The solution was stirred on a hot plate until the agarose is dissolved and the solution was clear and then solution was left to cool .
3. **Three** µl of ethidium bromide was added to the solution.
4. Gel chamber ends wereclosed with rubber gasket.
5. Acombs was pushed in the gel chamber about 1 inch from one ending of tray.
6. A gel solution was poured into the chamber and permitted to be harden for approximately 30 minutes at room temperature.
7. The combs were removed, and then samples and DNA ladder were loaded (4 µl) on each well with extreme cautions to avoid damages of the wells and cross contamination of neighboring wells .
8. The chamber is placed in a horizontal electrophoresis system and covered with the same TBE buffer that used to prepare the gel.
9. The cathode (black) was connected to the wells side of the unit and the anode (red) to the other side.
10. Electrophoresis is attach to direct current power source until dye markers migrated to the suitable distance, according to the size of DNA fragment that recognized.

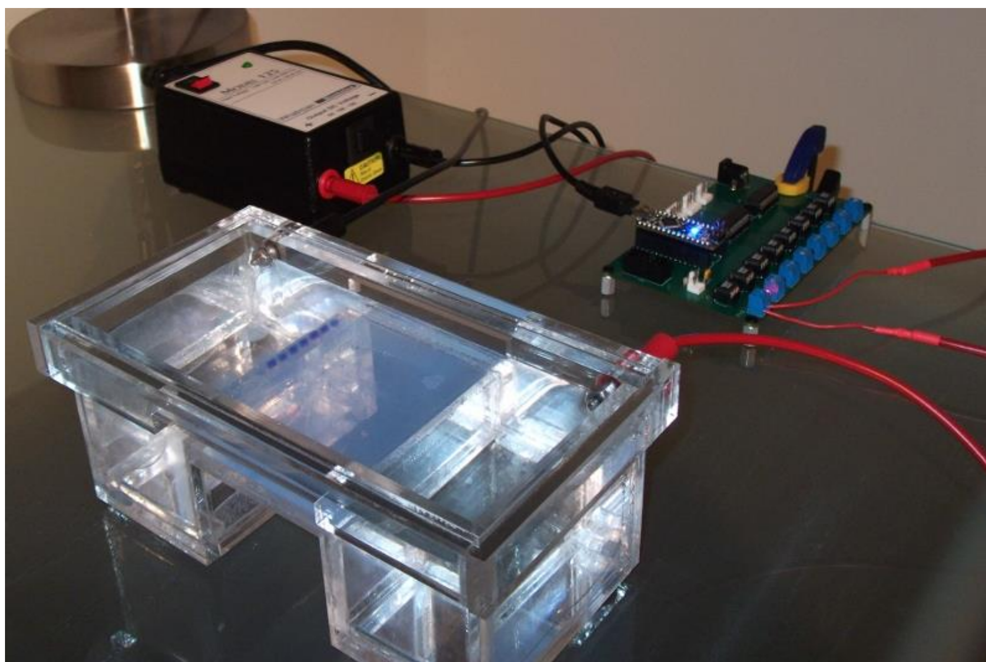


Fig. 2.5: Agarose Gel Electophoresis

2.5.2. DNA Electrophoresis

About 4µl of PCR product were loaded to each well with great precaution to prevent damages of the wells and cross contamination of neighboring wells. An electric field (50V for 35 min) was established to the system causing the negatively charged nucleic acids to travel across the gel to the positive electrode (anode).

2.5.3. DNA Ladder

In current study, 4µl of DNA ladder (1000 bp,Intron) was used as standard and band size ladder was 100- 1000 bp.

2.5.4. Gel - Band Visualization

To visualize the DNA bands, the agarose gel was placed in the UV trans illuminator device and exposed to UV light and the photos were captured by digital camera linked to PC.

CHAPTER THREE

Results
and
Discussion

3. Results and Discussion

3.1. The results of study groups

A total number of one hundred blood samples included in this study obtained from type 2 diabetic patients with/without ischemic heart diseases (IHD) were divided into two groups. The first groups include 50 patients with type 2 diabetes mellitus without IHD and the second group includes another 50 patients with type 2 diabetes mellitus with (IHD). The percentage age of patients besides non IHD groups was ranged between 18.0 - 28.0%, 34.0 - 30.0%, 28.0 - 34.0%, 4.0 - 0.0%, and 16.0 - 8.0% respectively as shown in figure 3-1, anova test were found to be 0.305, there was non-significant difference in age between patient have IHD and without IHD groups. This age matching helps to eliminate differences in parameters.

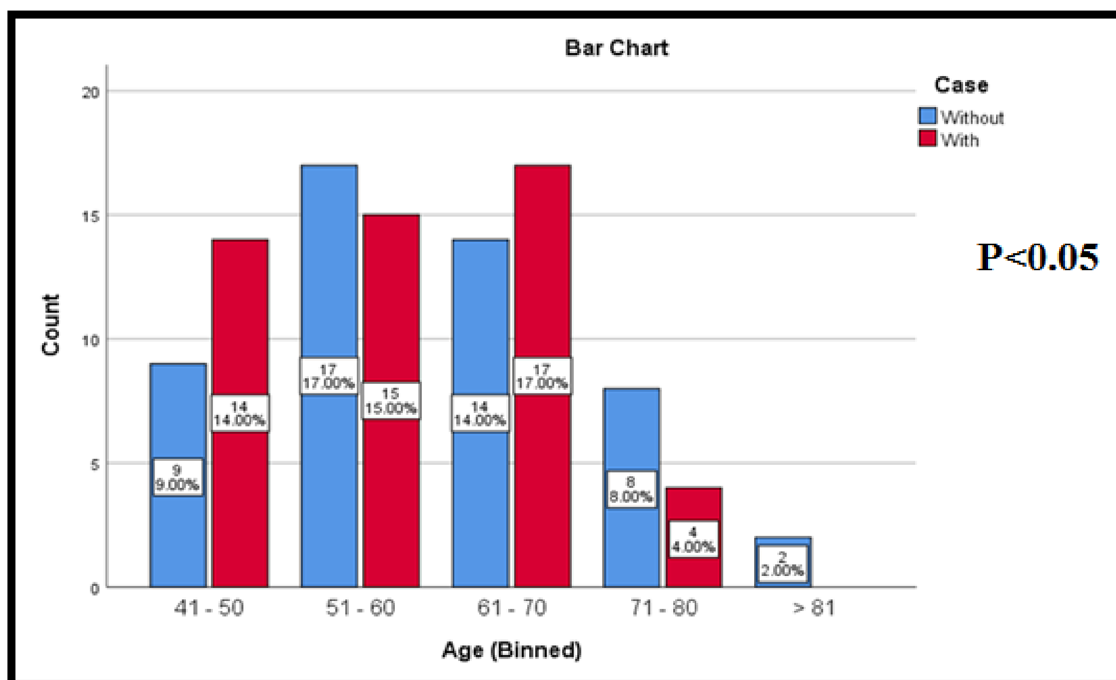


Fig. (3-1): Number and percentage of age in T2DM with/without IHD

However, the clinical details were non-significantly difference (P<0.05) between patients IHD besides non-IHD groups on gender table (3-1),all patient group study comprised of 46 females and 54 males, this result was differ with another study performed **(Maas and Appelman *et al.*, 2010)** who was found that cardiovascular disease affects women 7 to 10 years later than it does males, yet it remains the leading cause of mortality in women, due to the misunderstanding that women are "protected" from cardiovascular disease, the risk of heart disease in women is frequently overestimated. Because of the under-recognition of cardiac disease and the variations in clinical presentation between men and women, less aggressive treatment options are used and women are neglected in clinical trials. Furthermore, women's self-awareness and identification of their cardiovascular risk factors require more attention, which should lead to better cardiovascular event prevention **(Claassen *et al.*, 2012).**

Table (3-1): Number and percentage of type 2 diabetic patients with/without IHD according to gender status

Gender	Case of IHD		Total	*P.* value
	Without	With		
Female	22	24	46	0.421
	44.0%	48.0%	46.0%	
Male	28	26	54	
	56.0%	52.0%	54.0%	
Total	50	50	100	
	100.0%	100.0%	100.0%	

3.2. Relationship between BMI and Type 2 Diabetes Mellitus with/without Ischemic Heart Diseases

Table (3-2) shows the observed data of BMI in type 2 diabetic patients with IHD and without IHD patients. The mean ± SD of BMI in type 2 diabetic patients without IHD (31.6818 ± 4.42 kg/m^2) was non-significantly higher than that found in type 2 diabetic patients with IHD (30.0903 ± 4.99 kg/m^2) and the ($P > 0.05$). Some study demonstrated that obesity is a complex metabolic condition reported that affects 35% of the adult population in the United States, according to the National Institutes of Health. As a significant risk factor for ischemic heart disease (IHD) and its metabolic consequences, it has elevated to become one of the most serious health problems in many regions of the world **(McPherson *et al.*, 2015)**.

Table (3-2): Show BMI in type 2 diabetes mellitus with/without ischemic heart disease

		N	Mean ± SD	Mean ± SE	*P*. value
BMI	Without	50	31.6818 ± 4.420	31.6818 ± 0.63	0.094
	With	50	30.0903 ± 4.99	30.0903 ± 0.71	
	Total	100	30.886 ± 4.76	30.886 ± 0.5	

It was not noticed that a significant differences in body BMI between type 2 diabetic patients with/without heart disease and this results indicates the size of the sample used and the time obtained for blood samples. Although there are most studies that indicate a significant clinical relationship between body weight and heart disease **(Nordestgaard *et al.*, 2012).**

3.3. Biomarkers Studied in Type 2 Diabetic Patients with/without Ischemic Heart Disease

Table (3-3) shows the observed data of HbA1c in type 2 diabetic patients with IHD and without IHD patients. The mean ± SD of HbA1c% in type 2 diabetic

patients with ischemic heart disease was (9.674 ± 1.72%) which was slightly non-significantly higher than that found in patients without ischemic heart disease (9.64 ± 2.087%) (P = 0.921), this data was disagreement with other study **(Prasad, 2018)** who found that HbA1c was associated with cardiovascular disease (CVD), such as carotid and coronary artery atherosclerosis, ischemic heart disease, ischemic stroke, and hypertension, among other things. HbA1c causes dyslipidemia, hyperhomo-cysteinemia, and hypertension, as well as an increase in C-reactive protein level, oxidative stress, and blood viscosity, all of which are associated with the development of cardiovascular illnesses.

Table (3-3) shows the observed data of chromium metal in sera of type 2 diabetic patients with / without ischemic heart diseases. The mean ± SD of chromium determined in both group of patients studied indicated that the chromium level obtained in sera of type 2 diabetic patients with ischemic heart disease was significantly higher (12.45 ± 5.7 ppb) than its levels observed in T2DM without ischemic heart disease (4.99 ± 2.8 ppb) with ($P < 0.05$). In addition to its role in the control of insulin action, metabolic syndrome, and cardiovascular disease, chromium appears to have a protective effect on the body's immune system, an increasing body of evidence suggests that chromium may promote insulin signaling, and that supplementing with chromium may increase overall insulin sensitivity **(Hummel *et al.*, 2007)**, some study showed that plasma chromium levels in patients with coronary artery disease have been found to be significantly lower than in healthy subjects, according to recent studies, upon reviewing the study on the physiological functions of chromium, it becomes clear that the element has a role in nearly all of the known variables of cardiovascular risk **(Balk *et al.*, 2007)**, primarily through its effect on insulin levels and activities. Increased insulin levels are associated with chromium deficiency and are thought to be a main risk factor for cardiovascular disease; chromium insufficiency is associated with poor lipid and

glucose metabolism as well as increased insulin levels in the bloodstream **(Alissa *et al.*, 2009).**

Table (3-3) shows the results concerning the insulin level in sera of type 2 diabetic patients with / without ischemic heart diseases. The mean ± SD of insulin level determined in both group of patients studied indicated that its level in type 2 diabetic patients with ischemic heart disease was (6.86 ± 4.31 μU/mL) which non-significantly higher than that found in type 2 diabetic patients without ischemic heart disease is (6.03 ± 5.234 μU/mL) with P> 0.05. Cardiovascular illnesses are the leading cause of death worldwide **(Nowbar *et al.*, 2019)**, and type 2 diabetes is one of them because it is so common and doubles the risk of heart disease **(ERFC, 2010).** Increased glucose and insulin concentrations, as a result, have been proven to be pro-atherogenic in investigations **(Yu *et al.*, 2011).** Vafaeimanesh *et al*. in (2018) showed that cardiovascular diseases may be a consequence of insulin resistance rather than being caused by toxic effects of high insulin or glucose concentrations.

The mean ± SD level of HOMA-IR in type 2 diabetic patients with ischemic heart disease was non-significantly higher (3.351 ± 2.38) than that found in type 2 diabetic patients without ischemic heart disease (2.65 ± 2.41) with (P > 0.05), see table (3-3). Assessment of the homeostasis model insulin resistance (HOMA-IR) is a widely used and validated diagnostic of insulin resistance that includes both glucose and insulin concentrations. It represents insulin resistance, which can increase atherosclerosis through a variety of pathways **(Aydin and Ozkokeli, 2014)**. Insulin resistance has been linked to coronary artery disease in some studies.

Table (3-3): Show the level of HbA1c%, chromium, insulin, HOMA-IR and blood glucose concentration in T2DM with/without ischemic heart disease.

		N	Mean ± SD	Mean ± SE	*P*. value
HbA1c	**Without**	50	9.64 ± 2.087	9.64 ± 0.3	0.921
	With	50	9.674 ± 1.72	9.674 ± 0.243	

	Total	100	9.66 ± 1.9	9.66 ± 0.19	
Chromium, ppb	**Without**	50	4.99 ± 2.8	4.99 ± 0.4	0.000
	With	50	12.45± 5.7	12.45 ± 0.81	
	Total	100	8.72 ± 5.8	8.72 ± 0.6	
Insulin, μU/mL	**Without**	50	6.03 ± 5.234	6.03 ± 0.74	0.392
	With	50	6.86 ± 4.31	6.86 ± 0.61	
	Total	100	6.45 ± 4.79	6.45 ± 0.48	
HOMA-IR	**Without**	50	2.65 ± 2.41	2.65 ± 0.341	0.145
	With	50	3.351 ± 2.38	3.351 ± 0.34	
	Total	100	3.0 ± 2.41	3.0 ± 0.25	
FBG, mg/dL	**Without**	50	185.5 ± 56.77	185.5 ± 8.03	0.184
	With	50	198.9 ± 42.283	198.9 ± 5.98	
	Total	100	192.2 ± 50.256	192.2 ± 5.026	

Table (3-3) also shows the results concerning the fasting blood glucose level in sera of type 2 diabetic patients with / without ischemic heart diseases. The mean ± SD of FBG level determined in both group of patients studied indicated that its level in type 2 diabetic patients with ischemic heart disease was (198.9 ± 42.283 mg/dL) which is non-significantly higher than that observed in T2DM without IHD (185.5 ± 56.77 mg/dL), (P> 0.05). The impact of hyperglycemia on coronary heart disease (CHD) **(Doi *et al.*, 2010),** stroke **(Hyvärinen *et al.*, 2009),** and other cardiovascular diseases (CVDs) **(Levitan *et al.*, 2004)** has been widely studied. In people with hyperglycemia, two-hour plasma glucose (2hPG) is a better predictor of coronary heart disease (CHD) and ischemic stroke (IS) than fasting plasma glucose (FPG), but nothing is known regarding their impact in the normoglycemic range. Insulin resistance and beta cell dysfunction are already evident in people with increased normal FPG **(Lau *et al.*, 2019)**.

Table (3-4) shows that there is a non-significant differences in each of total cholesterol, triglycerides, low density lipoprotein-cholesterol and very low density lipoprotein-cholesterol in each of type 2 diabetic patients with/without ischemic heart diseases with the P>0.05. The mean ± SD of total cholesterol and TG level in sera of type 2 diabetic patients without ischemic heart disease were (221.04 ± 41.22 mg/dL and 212.7 ± 67.35 mg/dL) which is non-significantly higher than that obtained in patients with ischemic heart disease (216.96 ± 29.64 mg/dL and 199.2 ± 46.8) respectively. On the other hand the mean ± SD of each of lipoprotein HDL-C, LDL-C and VLDL-C levels in type 2 diabetic patients without ischemic heart disease were non-significantly slightly higher (40.994 ± 7.77 mg/dL ; 134.95 ± 25.27 mg/dL and 42.5 ± 13.47 mg/dL) than that found in type 2 diabetic patients with ischemic heart disease (38.32 ± 4.85 mg/dL ; 132.92 ± 21.91 mg/dL and 39.84 ± 9.36 mg/dL) respectively. HDL-cholesterol is inversely related to the risk of coronary heart disease and is an important factor in predicting cardiovascular risk despite its atheroprotection capabilities **(Lamarche *et* al., 1996).**

Independent of low-density lipoprotein-cholesterol, serum triglycerides (TG) and TG-rich lipoproteins (TGRL) have emerged as a risk factor for cardiovascular disease (CVD) (LDL-C) **(Sarwar *et al.,* 2007**), many prospective epidemiological studies demonstrate that while increased serum TGs are linked to coronary heart disease (CHD), this relationship is weakened after HDL-C and LDL-C are taken into account **(Li *et al.*, 2018**).

Table 3-4: Lipid profiles in sera of type 2 diabetes mellitus with/without ischemic heart diseases

		N	Mean ± SD	Mean ± SE	*P*. value
TC, mg/dL	**Without**	50	221.04 ± 41.22	221.04 ± 5.83	0.571
	With	50	216.96 ± 29.64	216.96 ± 4.2	
	Total	100	219.0 ± 35.78	219.0 ± 3.58	
	Without	50	212.7 ± 67.35	212.7 ± 9.5	0.248

TG, mg/dL	**With**	50	199.2 ± 46.8	199.2 ± 6.62	
	Total	100	205.92 ± 58.1	205.92 ± 5.81	
HDL-C, mg/dL	**Without**	50	40.994 ± 7.77	40.994 ± 1.1	0.042
	With	50	38.32 ± 4.85	38.32 ± 0.69	
	Total	100	39.66 ± 6.58	39.66 ± 0.66	
LDL-C, mg/dL	**Without**	50	134.95 ± 25.27	134.95 ± 3.57	0.670
	With	50	132.92 ± 21.91	132.92 ± 3.1	
	Total	100	133.94 ± 23.55	133.94 ± 2.36	
VLDL-C, mg/dL	**Without**	50	42.5 ± 13.47	42.5 ± 1.91	0.248
	With	50	39.84 ± 9.36	39.84 ± 1.324	
	Total	100	41.2 ± 11.62	41.2 ± 1.2	

There are some hypotheses that indicate that High-density lipoprotein (HDL-C) levels below 40 mg/dL are considered a major risk factor for coronary heart disease (CHD) and should be taken into account when making decisions about treatment of low-density lipoprotein (LDL-C) levels, according to national cholesterol treatment guidelines **(Keaney *et al.*, 2014)**, LDL-cholesterol is the primary contributor to artery-clogging plaque. HDL is a type of cholesterol that actively acts to remove cholesterol from the bloodstream. Triglycerides are another type of fat that circulates in our bloodstream. In recent years, research has revealed that excessive levels of triglycerides may potentially be associated with heart disease **(Blesso and Fernandez, 2018).**

3.4. Correlation between Various Biomarkers Studies among Type 2 Diabetic with/without Ischemic Heart Disease

Our result found a strong positive correlation between HOMA-IR and insulin level (r= 0.926) with significant differences (P<0.05) in the blood of type 2 diabetic patient with ischemic heart disease, insulin resistance occurs when cells do not

respond to insulin supplied through the bloodstream **(Lazar, 2006).** There are several reasons for this, there are occasions when a receptor has an issue, it's sometimes a problem with the signaling pathway, and the pancreas produces more insulin to compensate for inefficient insulin. Blood sugar levels will continue in the normal range as long as the amount of insulin supplied to the cell is sufficient to compensate for its weak response **(Petersen *et al*., 2007).**

The pancreas is frequently unable to maintain this enhanced insulin output for long periods of time, because insulin and sugar cannot be 'consumed' by the cells, there will be a lot of them in the blood, persistent hyperglycemia, or a high blood sugar level, has well-documented adverse implications, including diabetes and a higher risk of cardiovascular disease **(Fernández and Ricart, 2003).**

The result found indicate a negative correlation (r = -0.568) with significant differences ($P < 0.05$) between insulin levels and chromium in blood of type 2 diabetic patient with ischemic heart disease, it has been shown the use of chromium very important to reduce insulin resistance and to help to decrease the risk of cardiovascular disease and type 2 diabetes **(Rader, 2007)**. The chromium has been demonstrated to enhance insulin receptor kinase activity, as well as the activity of downstream insulin signaling effectors pI3-kinase and Akt, as well as Glut4 translocation to the cell surface.

Table (3-5): Correlation between Various Biomarkers among patients with ischemic heart disease

Correlations Type 2 diabetic patient with IHD						
		HbA1c	**Chromium**	**Insulin**	**HOMA-IR**	**FBG**
HbA1c	**Pearson Correlation**	**1**	**-0.317-***	**-0.193-**	**-0.051-**	**0.180**
	***P*. value**		**0.025**	**0.180**	**0.727**	**0.210**
	N	**50**	**50**	**50**	**50**	**50**

Chromium	**Pearson Correlation**	**-0.317-***	**1**	**0.568****	**0.477****	**-0.108-**
	***P*. value**	**0.025**		**0.000**	**0.000**	**00.456**
	N	**50**	**50**	**50**	**50**	**50**
Insulin	**Pearson Correlation**	**-0.193-**	**0.568****	**1**	**0.926****	**0.001**
	***P*. value**	**0.180**	**0.000**		**0.000**	**0.996**
	N	**50**	**50**	**50**	**50**	**50**
HOMA-IR	**Pearson Correlation**	**-0.051-**	**0.477****	**0.926****	**1**	**0.320***
	***P*. value**	**0.727**	**0.000**	**0.000**		**0.023**
	N	**50**	**50**	**50**	**50**	**50**
FBG	**Pearson Correlation**	**0.180**	**-0.108-**	**0.001**	**0.320***	**1**
	***P*. value**	**0.210**	**0.456**	**0.996**	**0.023**	
	N	**50**	**50**	**50**	**50**	**50**
***. Correlation is significant at the 0.05 level (2-tailed).**						
****. Correlation is significant at the 0.01 level (2-tailed).**						

Many studies showed that correlation between triglyceride and cholesterol with type 2 diabetic patient have ischemic heart disease (r = 0.737) with significant differences (P<0.05), this correlation goes back to increased triglyceride levels which may contribute to artery hardening or thickening (arteriosclerosis), therefore increases the risk of stroke, heart attack, and heart disease. Extremely high triglycerides can potentially induce severe pancreatic inflammation (pancreatitis) **(Tada *et al*., 2018).**

Obesity and metabolic syndrome, a cluster of disorders that includes too much fat around the waist, high blood pressure, high triglycerides, excessive blood sugar, and abnormal cholesterol levels - are typically associated with high triglycerides **(Girona *et al*., 2019).**

There was negative correlation (r = -0.682) between triglyceride and HDL-C in type 2 diabetic patient with ischemic heart disease with significant differences (P<0.05), this correlation indicated that the levels of HDL-cholesterol (HDL-C) in

the blood are inversely related to the levels of plasma triglycerides. The inverse association is connected to high-density lipoprotein (HDL-C) cholesterol and its dependence on the breakdown of triglyceride-rich particles by lipoprotein lipase (a lipoprotein lipase inhibitor) as shown in table 3-6. **(Peterson *et al.,* 2014).**

The study also found a negative correlation between total cholesterol level and HDL-C levels in type 2 diabetic patient with ischemic heart disease (r = -0.495), as a result of its location within lipoproteins, cholesterol moves throughout the body, known as "good cholesterol," HDL is responsible for transporting cholesterol to the liver, HDL assists in the removal of excess cholesterol from the body, and reducing the likelihood of it accumulating in the arteries **(Packard, 2018).** They are found positive correlation (r = 0.752) between triglyceride and LDL-C levels. The LDL-C (low-density lipoprotein), also known as "bad" cholesterol, accounts for the vast majority of the cholesterol in the body. Heart disease and stroke are more likely to occur if LDL cholesterol levels are elevated **(Lawler, *et al.,* 2017).**

Table (3-6): Correlation between lipid profiles among type 2 diabetic patient has ischemic heart disease

Correlations of type 2 diabetic patient with IHD					
		TC	**TG**	**HDL-C**	**LDL-C**
TC	**Pearson Correlation**	1	.737**	-.495-**	.752**
	***P*. value**		.000	.000	.000
	N	50	50	50	50
TG	**Pearson Correlation**	.737**	1	-.682-**	.802**
	***P*. value**	.000		.000	.000
	N	50	50	50	50
HDL-C	**Pearson Correlation**	-.495-**	-.682-**	1	-.692-**
	***P*. value**	.000	.000		.000
	N	50	50	50	50
LDL-C	**Pearson Correlation**	.752**	.802**	-.692-**	1

	***P*. value**	.000	.000	.000	
	N	50	50	50	50
VLDL-C	**Pearson Correlation**	.737**	1.000**	-.682-**	.802**
	***P*. value**	.000	.000	.000	.000
	N	50	50	50	50
**. Correlation is significant at the 0.01 level (2-tailed).					

3.5. Prediction of some Biomarkers during Measurement in the Type 2 Diabetic Patient with Ischemic Heart Disease.

The ROC curve analysis is used by medical experts to investigate diagnosis performance, the area under the curve (AUC) is used to measure the ROC plot (AUC). The ROC plots were used to evaluate the performance of each categorized blood parameter value. This was estimated using the area of the ROC curve, also known as the area under the curve (AUC) table (3-7). The curve's value is between 0 and 1and indicates the model's overall reliability; a value of 1.0 indicates great sensitivity and specificity (**Siddiqui *et al*., 2020).** In this case, the AUC is utilized to assess the accuracy of each parameter category. An AUC of 1.0 suggests that the following test findings, such as chromium status in the data set, can be predicted without error. An AUC of 0.50, on the other hand, indicates a 50% chance of accurately predicting insulin, HbA1c, HOMA-IR and FBG categories. As shown in the picture, the stronger classifier should be set near the left corner of the ROC plot's height figure (3.2.).

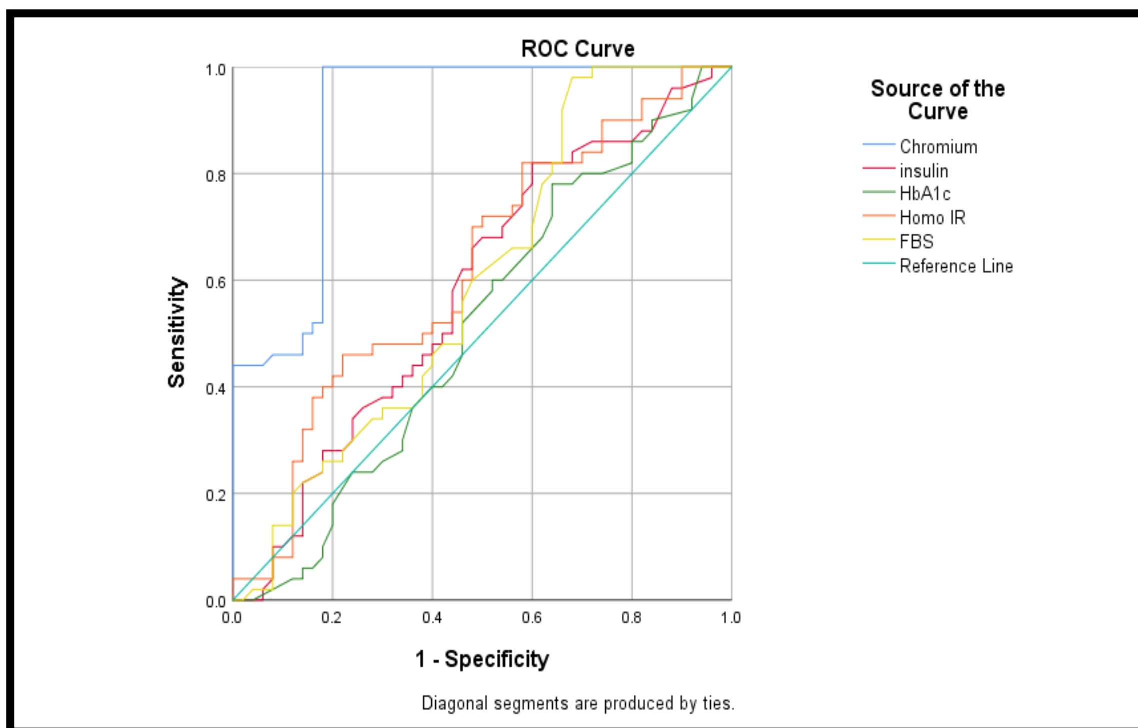


Fig. (3-2): ROC curve analysis and the true positive rate (TPR) and false negative rate (FPR) are plotted on a two-dimensional graph, for prediction of some blood parameters.

Table(3-7): Area under the curve to analysis of some blood parameters in the type 2 diabetic patent with ischemic heart disease

Area Under the Curve					
Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Chromium	o.903	0.032	0.000	0.841	0.966
Insulin	0.584	0.057	0.150	0.471	0.696
HbA1c	0.513	0.059	0.828	0.398	0.627
HOMA-IR	0.622	0.056	0.035	0.512	0.733
FBG	0.596	0.058	0.099	0.483	0.709

The test result variable(s): Chromium, insulin, HbA1c, FBG has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption
b. Null hypothesis: true area = 0.5

When the chromium level is less than 4.25, the individual is classified as having cardiovascular disease. The element chromium has a potential association with ischemic heart disease and has been designated as a prediction marker **(Abebe *et al.*, 2010)**. Ischemic heart disease is associated with serious health difficulties such as atherosclerosis, myocardial ischemia, health-related behaviors, and other biological risk factors. As indicated in Table (3-7), it was found that the AUC value was 0.903, which is more than 0.5, indicating that the chromium level could be predicted with a high degree of accuracy (95.76 %) and indicating a better effect.

3.6. The Relationship between Multigenerational Impact of the MTTL1 A3243G with type 2 Diabetic Patients with Ischemic Heart Disease.

we screened 100 patients of type 2 diabetes mellitus with / with out schemic heart disease to detected 3243 A/G mtDNA mutations.

The amplification product of MTTL1 Gene polymorphism(SNP of A3243G) detected by allele specific PCR reaction, have a size of 422 bp.

Our result concerning the mitochondrial DNA found that just only four cases has G allele of the MTTL1 A3243G gene in type 2 diabetic patient with ischemic heart disease, and only one case without ischemic heart disease was observed as indicated in figure (3.4). The study showed that the G allele is responsible for heart disease. It was reported in 2010 that individual's carrying the MTTL1 A3243G gene mutation have been diagnosed with ischemic cardiac disease **(Tuppen *et al.,* 2010).**

The MTTL1 A3243G gene mutation affects mitochondrial DNA structure, stability, methylation, amino-acylation, and codon recognition capabilities, making it difficult to couple the mRNA codon with the mutant tRNA anticodon **(Finsterer,**

2007). This condition is most commonly linked to an A to G transition in the mitochondrial DNA at location 3243 and incorrect RNA processing results in reduced translation as well as decreased rates of protein synthesis and enzyme activity. A statistically significant negative correlation was observed between the frequency of MTTL1 A3243G mutations and the particular activity of the mitochondrial respiratory chain complex **(Figure 3-2).** It has been discovered that the respiratory chain complex with the highest amount of mitDNA-encoded subunits were exists.

The result observed in this study found that the mean of Homeostatic Model Assessment for Insulin Resistance in the patient without ischemic heart disease have A allele in their blood (2.88 ± 1.63), while the HOMA-IR evaluated in type 2 diabetic patient with ischemic heart disease was (3.34 ± 1.95), on the other hand, our result found one case with Homeostatic Model Assessment for Insulin Resistance in the type 2 diabetic patient without ischemic heart disease have G allele in his blood (1.1141), and it was recorded as (4.239 ± 1.76) in diabetic patient with ischemic heart disease table (4-8).

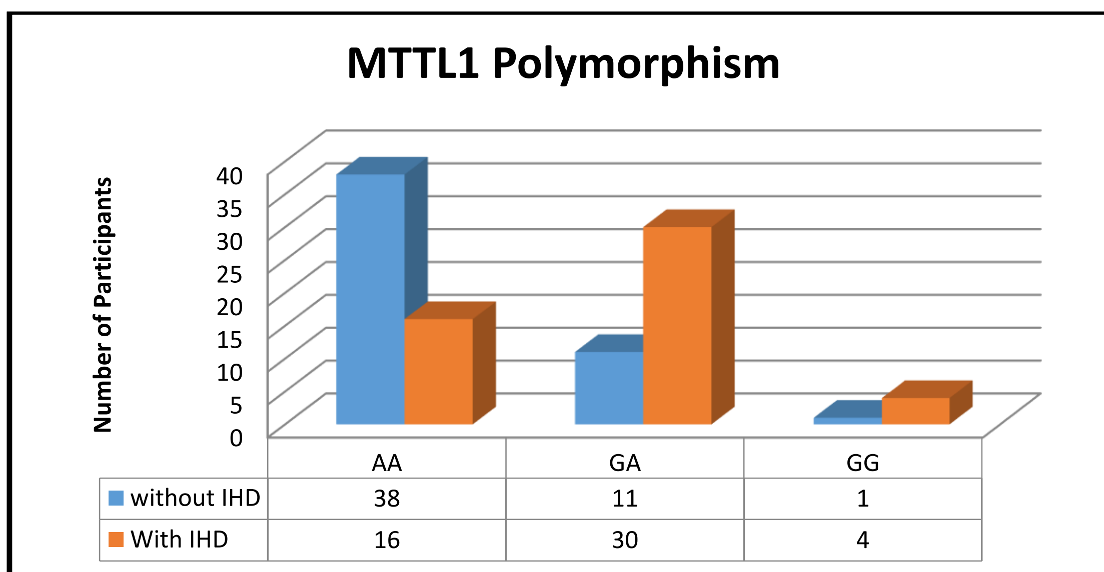


Fig. (3-3): The relationship between Multigenerational Impact of the MTTL1 A3243G with Homeostatic Model Assessment for Insulin Resistance

The genotype of GG in multigenerational Impact of the MTTL1 A3243G increases the risk of HOMA-IR in mitochondria and their genome are found in the cytoplasm of cells, with thousands of copies of the mitochondrial genome in most cell types. Heteroplasmy occurs when not all copies of the mitochondrial genome have the same sequence at the tissue or even cellular level (resulting in different proportions of mutant and wild type mitochondria) **(Stefano *et al.*, 2017).**

When mitochondria are randomly segregated to each new cell during cell division, the fraction of mitochondrial DNA containing a mutation may vary between daughter cells as a result of heteroplasmy. When the load or proportion of mitochondrial DNA with a harmful mutation exceeds a certain threshold level, tissues show pathogenic effects of mutation **(Chen *et al*., 2020).**

Table (3-8). The relationship between Multigenerational Impact of the MTTL1 A3243G with HOMA-IR.

T2DM	Gene	Mean ± SD	N	P. value
Without IHD	**AA**	2.881 ± 1.64	38	0.013
	GA	1.974 ± 1.33	11	
	GG	1.114 ±	1	
	Total	2.646 ± 2.410	50	
With IHD	**AA**	3.344 ± 1.951	16	
	GA	3.236 ± 2.68	30	
	GG	4.24 ± 1.762	4	
	Total	3.351 ± 2.382	50	
Total	**AA**	3.02 ± 2.45	54	
	GA	2.897 ± 2.442	41	
	GG	3.615 ± 2.07	5	
	Total	2.999 ± 2.411	100	

As illustrated in Figure (3.4.), the successful amplification and analysis of the SNP of Multigenerational Impact of the MTTL1 A3243G was achieved using the A3243G. Detection of PCR bands of suitable size in the agarose gel indicated that the samples were of the appropriate genotype as shown in Figure (3-4).

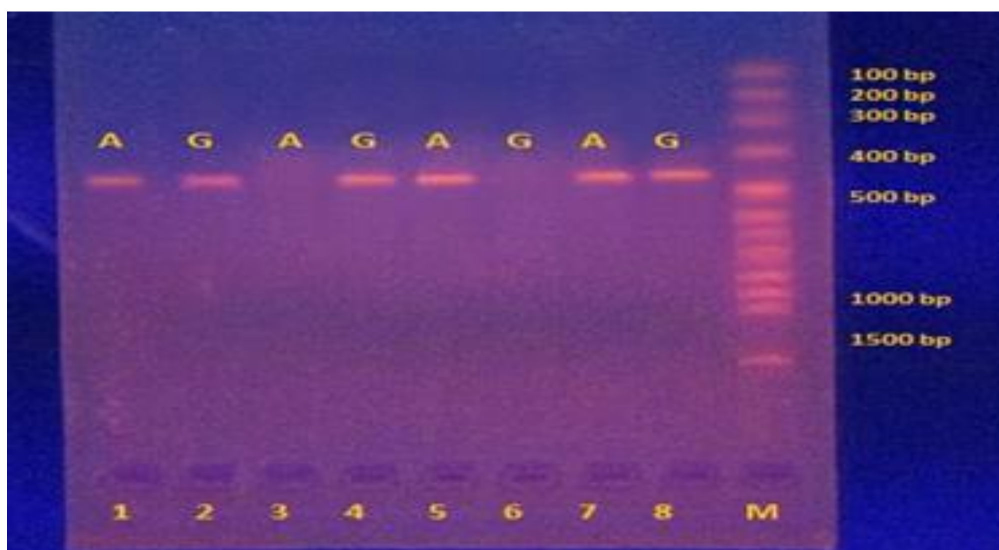


Fig. (3.4): The electrophoresis profiles for some of the successful amplifications. Multigenerational Impact of the MTTL1 A3243G .

M = Lane for DNA ladder marker

1,2 = Lane for hetrozygote patient

3,4 = Lane for G allele patient

5,6 = Lane for A allele patient

7,8 = Lane for hetrozygote patient

Total 422 nucleotides containing DNA was amplified by polymerase chain reaction (PCR), The forward primer was taken from nucleotide sequence 3035 to 3054 as 5′ - GCA AGA GAT ACA GTG TTG CTC CA -3′ and the reverse primer was taken from nucleotide sequence 3437 to 3456 as 5′- CGT TCT CTA TGT CAC AAC GAG

GT -3′ After electrophoresis, absence of the mutation generates a single band (422 bp).

The amplification product of MTTL1 Gene polymorphism(SNP of A3243G) detected by allele specific PCR reaction, have a size of 422 bp. The PCR product was electrophoresed on 1% agarose and directly was visualized with ethidium bromide under UV light. . The amplification and the size of the resulted amplicons were confirmed by agarose gel electrophoresis analysis .

4.1. Conclusion:

1. The SNP (A 3243G) of the MTLL1 gene was associated with increased the risk of DMT2 with ischemic heart disease in Iraqi patients and it is not significant with DMT2 with out ischemic heart disease.

2. A significant positive correlation was found between chromium serum level and HOMA-IR in T2DM with ischemic heart disease .

3. A significant association was found between biochemical and molecular parameter in DM patients .

4. The genotype of GG in Multigenerational Impact of the MTTL1 A3243G increases the risk of HOMA-IR Mitochondria and their genome are found in the cytoplasm of cells, with thousands of copies of the mitochondrial genome in most cell types.

5. mtDNA polymorphism can be used as a marker for the genetic elucidation of the world population; hence the detection of single nucleotide polymorphism in mtDNA has attended much prominence.

4.2. Recommendation

1. Future research is required to well understanding the role of all mt DNA gene polymorphisms with Diabetic mellitus in Iraqi population.

2. Analysis of more SNPs of *MTLL1* gene to determine which one is more common in Iraqi population.

3. Study the relations between the pathogenesis of *MTLL1* and immunomarkers such as Anti islet insulin and Anti GAD in DMT1.

4. Study design about follow up relation between HbA_1C level and chromium ion before taking chromium supplement and after .

5. designind a study for those interested in keto diet and determine chromium ion level with lipid profile because those people used large amount of special tybe of carbshydrat in diet (commen name called stevia).

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الخلاصــــــة

داء السكري هو اضطراب ايضي يتميز بوجود فرط سكر الدم المزمن مصحوبًا بضعف أكبر أو أقل في أيض الكربوهيدرات والدهون والبروتينات .أحد المضاعفات المهمة لمرض السكري هو مرض القلب ذات التورية القليله لهذه الدراسة في محاولة لدراسة الارتباطات للعديد من العلامات الحيوية المتغيرة والطفرة النوعية مقارنة بالمرضى الذين يعانون من أمراض القلب ذات التورية القليلة ، وكانت دراستنا عبارة عن دراسة مقطعية علينا اختيار مجموعتين دراسيتين المجموعة الأولى من داء السكري من النوع الثاني مع أمراض القلب ذات التورية القليله (٥٠ مريضاً) ، والمجموعة الثانية من داء السكري من النوع ٢ غير المصابين بأمراض القلب ذات التورية القليلة (٥٠ مريضاً) باستخدام انبوب ال EDTA ، تم سحب عينة الدم من كل مريض ، ثم تم استخراج mit DNA بواسطة محاليل متخصصه. ثم تم mit DNA المستخرج من مصل داء السكري من النوع ٢ مع وبدون أمراض القلب الإقفارية باستخدام تقنية تفاعل البوليميراز المتسلسل للتحقيق في الطفرة المحددة في زوج القاعدة (٣٢٤٣) التي تحدث في mit DNA من أجل التفريق بين أمراض مختلفة .وجدت نتيجتنا ارتباطًا إيجابيًا قويًا بين مقاومة الانسولين والأنسولين (= ٠,٩٢٦) مع وجود فروق ذات دلالة إحصائية (0.05>) في دم المريض المصاب بمرض نقص تروية القلب ، ووجدت

النتيجة ارتباطًا متوسطًا (٠,٥٦٨) مع فروق ذات دلالة إحصائية (٠,٠٥) بين مستويات الأنسولين مع وجود الكروم في دم المريض يعاني من أمراض القلب ذات التورية القليله ، عندما يكون مستوى الكروم أقل من ٤,٢٥ ، يصنف الفرد على أنه مصاب بأمراض القلب والأوعية الدموية. عنصر الكروم له ارتباط محتمل بأمراض القلب الإقفارية وقد تم تحديده كمؤشر تنبؤ .تضخيم يعطي الجين نمطًا وراثيًا واحدًا كما هو موضح بواسطة نطاقات (٤٢٢)لأولئك الذين لديهم نوع بري متماثل (AA) ، وأنماط وراثية متحولة متماثلة اللواقح (GG) ونطاقين من الأنماط الجينية (٤٢٢ نقطة أساس) لمن لديهم زيجوت متغاير الزيجوت .(GA) تم العثور على ترددات النمط الجيني لتعدد الأشكال A3243G لتكون متوافقة مع توازن هاردي واينبرغ. ترددات الأليل (٣٢٪ ، ٥٨٪ ، ١٠٪) من (AA,GG,GA) في حالات داء السكري مع مجموعة أمراض القلب الإقفارية و (٧٦٪ ، ٢٢٪ ، ٢٪) من (AA,GA,GG) في حالات مرض السكري دون الإصابة بمرض نقص تروية القلب .يبلغ مستوى الكروم ٤,٩٩٠٠ في الدم بدون أمراض القلب الإقفارية ومستوى الكروم ١٢,٤٤٦٠ في الدم المصابين بأمراض القلب الإقفارية وهذه النتائج معنوية (٠,٠٥). بالإضافة إلى دوره في السيطرة على عمل الأنسولين.

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دور الحامض النووي للمايتوكوندريا DNA , أيون الكروم و علامات كيموحيوية في النوع الثاني لداء السكري مع و بدون أمراض القلب الإقفارية

رسالة

مقدمة الى مجلس كلية الطب - جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في (الكيمياء السريرية)

من قبل

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