STUDY 0F CLINICAL CHARACTERISTICS AND ASSOCIATION OF A CDKAL1 GENE POLYMORPHISM (RS10946398) WITH DIABETES MELLITUS TYPE 2 T2DM IN CASES PRESENTED AT THE DIABETIC CENTRE ISLAMABAD

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2023

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A dissertation submitted in partial fulfillment of the requirements for the degree of
Master of Philosophy
In
Molecular Biology

By

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Islamabad

2023

In the name of Allah Almighty, The Most Gracious, The Most Beneficent, The Most Merciful

CERTIFICATE

This dissertation *"Study of Clinical characteristics and association of a CDKAL1 gene polymorphism (rs10946398) with Diabetes Mellitus T2DM in cases presented at The Diabetes Centre ,Islamabad."* submitted by ***Zainab Fida***, is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-I-Azam University, Islamabad, Pakistan as satisfying the thesis requirement for the degree of Master of Philosophy in Molecular and Cell Biology.

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DECLARATION

I hereby declare that the work presented in this thesis is the result of my own efforts and research work, carried out in Molecular Biology Lab, Department of Zoology, Quaid-I-Azam University Islamabad.

This thesis is my own composition and no part of it has been presented for any degree previously, nor does it contain, without proper acknowledgement or reference, any material from the published resources to the best of my knowledge.

Zainab Fida

DEDICATED

TO

MY BELOVED PARENTS AND MY HUSBAND WHO HAVE BEEN PILLARS OF SUPPORT,

ACKNOWLEDGEMENT

All the praise and thanks to **Almighty Allah** for his countless blessings and for giving me strength to complete my research work successfully. Blessings of Allah be upon His **Holy Prophet (PBUH)** who brought us out of the darkness and enlightened the way to Heaven, who has guided His Ummah to seek knowledge from Cradle to Grave. All the glory and thanks to Almighty Allah for His countless blessings and for giving me the potency to finalize my research work successfully.

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LIST OF ABBREVIATION

Abbreviation	Full Forms
T2DM	Type 2 diabetes mellitus
WHO	World health organization
FFA	Free fatty acids
BMI	Body mass index
HbA1C	Hemoglobin A1c
TNF-α	Tumour necrosis factor alpha
IFN-γ	Interferon
PKC	Protein kinase C
ER	Endoplasmic reticulum
tRNA	Transfer RNA
SNP	Single nucleotide polymorphism
IR	Insulin receptor
tRNA	Transfer RNA
ARMS	Amplification Refractory Mutation System PCR
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic Acid
TNE	Tris-CL NACL EDTA
TBE	Tris-borate-EDTA
PCI	Phenol chloroform isoamylalchol
SDA	Sodium dodecyl sulfate
EDTA	Ethylenediamineteraacetic acid
T-EDTA	Tris-EDTA
dNTP	Deoxynucleotide
MgCL2	Magnesium chloride
KPK	Khyber Pakhtun kawa
HB	Hemoglobin
CDKAL1	CDK5 regulatory subunit associated protein 1 like 1
KCNQ1	Potassium voltage-gated channel subfamily Q member1
TCF7L2	Transcription factor 7 like 2
TDC	The Diabetes Centre

CTD	C-terminal Domain
SSD	Sterol Sensing Domain
LAL	Lysosomal Acid Lipase
DBS	Dried Blood Spots
MS	Mass Spectrometry
LDL	Low Density Lipid
ACAT	Acyl CoA: Cholesterol Acyl Transferase
7-KC	7-ketocholesterol
C-triol	5β-cholestan-3β,5α,6β-triol
Lys-SM 509	Lysosphingomylin-509
CT	Chitotriosidase
CVS	Chronic Villus Sampling
SRT	Substrate Reduction therapy
FDA	Food and Drug Authority
CD	Cyclodextrin
ERT	Enzyme replacement therapy
AAV	Adeno Virus-Associated Virus
IRE	International Review Board
GIT	Gastrointestinal Track (GIT)
I.Q	Intellectual Quotient
CBC	Complete Blood Count
LFT	Liver Function Test
RFT	Renal Function Test
MRI	Magnetic Resonance Imaging
EDTA	Ethylenediaminetetraacetic Acid
PCR	Polymerase Chain Reaction
Rmp	Revolutions Per Minute
PK	Proteinase K
SDS	Sodium Dodecyl Sulphate
TE	Tris EDTA
TBE	Tris Borate EDTA

ABSTRACT

Diabetes Mellitus type 2 is a prevalent disease that afflicts more than 415 million people and a leading cause of morbidity and moratility worldwide. It is influenced by both the genetic and non -genetic factors. Among non-genetic risk factors age, BMI, lack of physical activity, obesity, hypertension, and many other factors contribute to its prevalence.CDK5 Regulatory subunit association protein 1 *(CDKAL)* is a protein encoding gene which encodes a tRNA modifying enzyme which is involved in the proper protein translation and in the regulation of insulin production, encoded by *CDKAL1* gene variations in the *CDKAL1* gene sequence leading to the misreading of Lys codon in proinsulin, which results in the decreased production of glucose stimulated pro-insulin. In present study a total of 100 types 2 patients were enrolled through THE DIABETES CENTRE Islamabad. Following the study the possible demographic and clinical risk factors were documented and genotyped to study gene association. Various sequence of *CDKAL1* are polymorphisms such as r s4712523, rs10946398, rs7754840, and rs7756992,rs9465871 reported to be associated with T2DM of these variants the SNP rs10946398 is reported to have an impact on risk of type 2 diabetes in different population including European , Japanese, Chinese and Indian population. Research have shown that this SNP rs10946398 overturns the regulation of *CDKAL1* expression, results in decreased insulin production however there is no reported case from our population. Blood samples collected from the patients after informed consent. DNA was extracted and the primers were designed to perform the TETRA-ARMS PCR. In our study we genotyped 17 T2DM patients and 10 controls for rs10946398 *CDKAL1* using tetra ARMS PCR. Data analysis of clinical and demographic characteristics revealed that among diabetes patients most of the cases were males with age higher than 35y.80% of the Patients had cholesterol more than 150mg/dl , 94% had HBA1C more than 6 , 76.6% had higher triglyceride levels more than 150mg/dl. After TETRA ARMS PCR Analysis the AA allele was found to be 8 in cases and 6 in control AC was 7 in cases and 3 in control however minor allele (C) was found in homozygous form in 2 cases and 1 control. As number of cases genotyped for r s 10946398 polymorphism was not sufficient therefore showed a non- significant association between (p=0.25) with risk of type 2 diabetes. Therefore the findings of the C allele for DM in our population could not be ruled out. Hence study do not support *CDKAL1* gene polymorphism rs10946398 in Pakistani population. However this was a small scale study further large studies are required to

find the exact probability of the variant in a population and its role in disease onset and its progression and pathogenesis.

INTRODUCTION

Diabetes mellitus is a metabolic disorder defined by high amount of glucose in blood. People suffered from diabetes may develop other serious health issues as a result low quality of life, increased expenses and high mortality. High levels of glucose in blood can affect kidneys, heart, eyes and other organs as a result of vascular damage.

Over the past years the pace of impaired glucose and diabetes has been increased globally. This increased prevalence of diabetes is due to urbanization and the inactive lifestyle in many countries.

Diabetic patients have an increase risk of mortality from various infections like COVID 19, kidney infection, pneumonia and foot diseases. (Magliano, D. J.*et al,.,*2022)

Diabetes is increasing rapidly worldwide and it is estimated that it can affect about 693 million people by 2045. & (Florez, J. C *et al.,*2020)

Diabetes mellitus type 2 complications;

It is a chronic, multifunctional disease which affects different body organs. High level of glucose in blood can affect microvasculature which results in diabetic retinopathy, diabetic nephropathy, and neuropathy result in low life expectancy and overall quality of life. About 25% of the diabetic patients affects with diabetic nephropathy and diabetic retinopathy. Of all the diabetic patients 50% accounts for diabetic neuropathy. The common risk factors for the development of these complications are glycaemia, blood pressure and lipid control. (Dimitriadis, K.*et al.,*2020).

Signs and symptoms of diabetes type 2

Among older adults the symptoms include

- Hypoglycemia
- Fatigue
- Pain
- Diarrhea
- Loss of balance and
- Falling

(Chesla, C. *et al*., 2019)

Global prevalence of diabetes;

Over the past few decades, adult around the world have seen an increase in the pace of diabetes and impaired glucose tolerance [3], [4], [5], [6]. Rapid population growth and radical shift towards the inactive lifestyles have driven up the diabetic prevalence in many nations and areas. For the purpose of allocating community and health resources and as well as for the development of initiatives to combat these increasing tendencies exact figures of the existing and upcoming burden of diabetes are required.

Global prevalence of Diabetes mellitus type 2

The prevalence rate of 6059 cases per 100,000 people was reported in 2017 affecting 462 million people roughly. And the age of population was among 4.4% between15 to 49, 50 to 69 were 15% and over 70 years were of 22%. Annually 10 million deaths caused due to diabetes therefore considered as the 10^{th} largest cause of death. There is worldwide occurrence of the disease and in western Europe it is increasing efficiently. Most of the patients lie in age 55 and number found equally in both males and females. By 2030, it is anticipated that there would be 7079 people worldwide with type 2 diabetes for every 100,000 people, depicting that the trend would follow in all geographical regions. 462 million people are expected to be affected by diabetes, or 6.28% of the world's population. It was considered as 9^{th} biggest cause of death with 1 million deaths reported in 2017. This situation of increasing numbers is alarming compared to data in 1990 when type 2 diabetes was listed as the 18th biggest reason of death. In terms of DALYs, it is the 7^{th} most prevalent disease which measure human misery (Al Kaabi, J. *et al*.,2020).

Disease burden of type 2 diabetes, 2017

Table 1.1 showed the diseased burden of diabetes type 2

Region	Prevalence (cases per 100,000)	Burden of suffering (DALY per 100,000)
Global	6059	751
Europe	8529	842

Region	Prevalence (cases per 100,000)	Burden of suffering (DALY per 100,000)
Germany	9091	820
France	6843	564
Italy	9938	1083
Spain	8796	773
Netherlands	11,344	924
Switzerland	10,040	815
Sweden	10,448	877
Turkey	6483	889
Russia	6865	740
United Kingdom	8663	644
Asia	5961	729
China	6262	635
India	4770	663
Japan	6737	553
South	8835	

Region	Prevalence (cases per 100,000)	Burden of suffering (DALY per 100,000)
Korea		

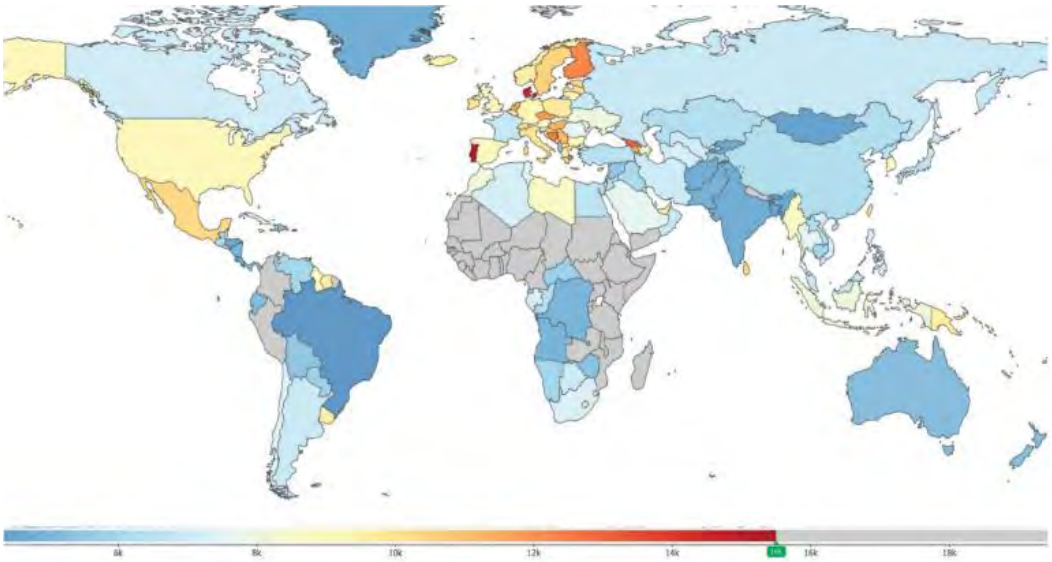

Figure 1.1 Global distribution of diabetes type 2. Colors indicate prevalence rates per 100,000.

Paradoxically, certain areas—like the island nations in the Pacific Ocean—have the greatest rates of sickness. Fiji (20,277 per 100,000), Mauritius (18,545), American Samoa (18,312), and Kiribati are among these nations (17,432). In the past two decades, Southeast Asian nations including Indonesia, Malaysia, Thailand, and Vietnam have advanced. China (88.5 million people with type 2 diabetes), India (65.9 million), and the US (28.9 million) continue to hold the top ranks as the nations with the highest overall numbers of people with this ailment due to their vast populations. (Al khaabi.e.,al 2020)

In Females it has been seen that they have lower prevalence of the disease than males (6219 cases per 100,000 versus 5898), however this range is still uncertain.Incidence peaked between the ages of 55 and 59, but the age of the disease diagnosis in famele is late and in males it is slightly earlier and exhibits expected patterns of increased pace would occur with increasing age (Figure 3). But this doesn't seem a significant shift in age distribution from 1990 to 2017.

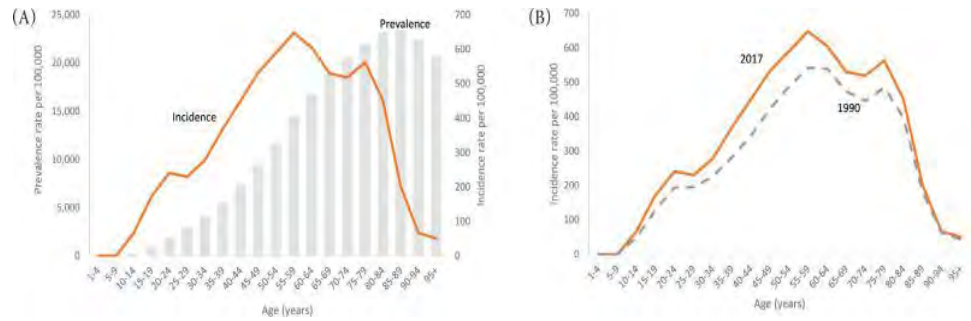

Figure 1.2 shows the age distribution of type 2 diabetes mellitus globally. (A) Prevalence vs. incidence (both 2017). (B) The prevalence in 1990 and 2017. Chi-square test: p 0.0001.(Mustafa, H *e.al*,.2020)

Prevalence of the disease in Pakistan;

In Pakistan, type 2 diabetes mellitus still predominates (11.77%). Around 11.20% of men and 9.19% of women are dominant, respectively .In Punjab territory, it is 12.14% for men and 9.83% for women; in Sindh, it is 16.2% for men and 11.70% for women. In Baluchistan the prevalence is more in males than females. Female prevalence is 8.9% while male prevalence is 13.3% However, male prevalence is 9.2% and female prevalence is 11.60% in KPK. The predominance is higher in males than in females. Diabetic preventive measures should be considered by Pakistan.

Risk factors for diabetes;

According to past national and international studies, age plays a significant role in the increased risk of pre-diabetes and diabetes. Age had a substantial impact on the likelihood of pre-diabetes after other potential variables were eliminated.

Older adults are more likely to have pre-diabetes and diabetes than younger adults are. This finding is consistent with those of earlier studies. A lesser degree of education, on the other hand, also showed to be a risk factor for both pre-diabetes and diabetes. Individuals with higher levels of education typically had a better awareness of the condition and were able to effectively control their blood sugar. Also, the different levels of education had an impact on the participants' employment and income, which in turn had an impact on their health.

BMI-based overweight and obesity are risk factors for developing diabetes and pre-diabetes. Prior studies have demonstrated that a high BMI increases the likelihood that an older person would develop T2DM, supporting the findings of our study. The senior population is also more prone to early onset of obesity and higher

insulin resistance than the middle-aged group, which raises the risk of diabetes. This is because of advanced age, hormonal changes, and an inactivity.

Diabetes and dyslipidemia are strongly associated, therefore people with type 2 diabetes frequently have low HDL, elevated TG, and raised levels of tiny, dense LDL. Like a serum biomarker, TG can be used to forecast the likelihood of developing diabetes; as a result, elevated TG levels raise the possibility of developing pre-diabetes. While ageing raised blood TG levels and altered the body's TG metabolism, elevated TG levels in our study only increased the risk of pre-diabetes and diabetes in the old group. As a result, metabolic illnesses like diabetes, and non-alcoholic fatty liver disease and metabolic diseases or syndromes were known to be at higher pace in older persons than in younger ones. (Lu, H.*e al.,(*2023).

Pathophysiology of the disease;

B cell dysfunction and insulin Resistance;

In both the pathophysiology and the onset of type 2 diabetes, insulin resistance and -cell dysfunction play significant roles. (Kahn, S. E.*e.al* 2002).

The presence of beta-cell dysfunction in Type 2 diabetic individuals is unmistakable. This change can be seen in a variety of ways, such as to intravenous glucose reduce response of insulin. [68, 69, 70] and a decrease in glucose's capacity to enhance the insulin response to non-glucose signaling molecules like the amino acid arginine [71], hormones like secretin [72], the beta-adrenergic agonist isoproterenol [72], and sulfonylureas like tolbutamide. Alterations in ultradian oscillatory insulin secretion [76] and pulsatile insulin release [74, 75] can also be seen. Moreover, the beta cell cannot swing in time with the changes in plasma glucose brought on by an oscillating glucose infusion [76]. The ineffective conversion of pro insulin to insulin results in amyloid peptide. (Kahn, S. E. (2003) Figure 1

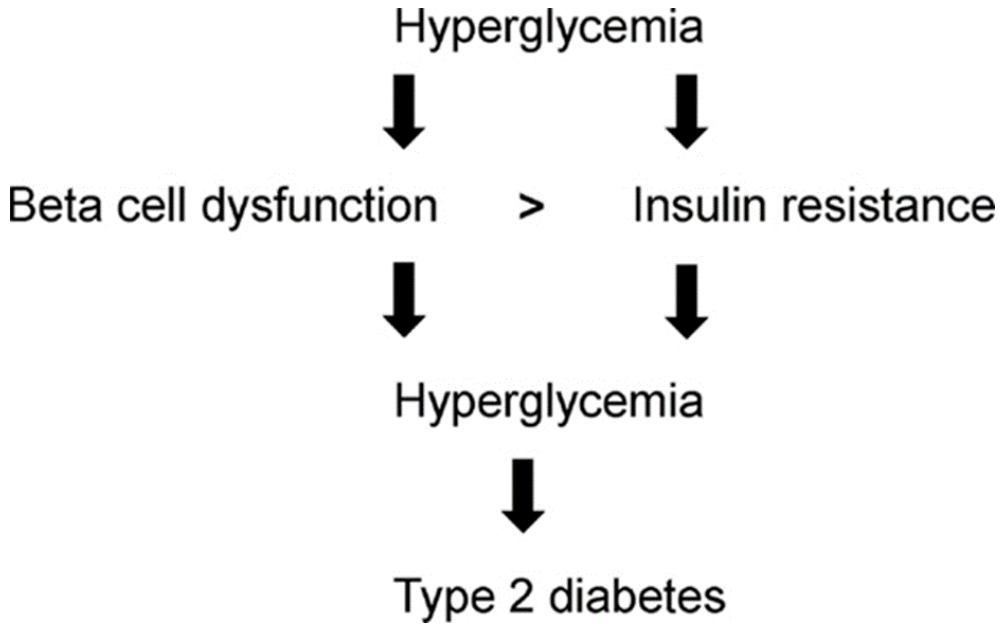

Figure 1.3 Hyperglycemia-Induced Beta Cell Dysfunction, Insulin Resistance, And Type 2 Diabetes. Cerf, M. E. (2013)

Inflammation in adipose tissues;

Inflammation in adipose tissues is also caused by over nutrition and obesity, which is considered as the underlying cause to several obesity-related diseases includes insulin resistance and T2D, cancer, atherosclerosis. Chronic inflammation of adipose tissue is caused by the infiltration of macrophages, increase of immune populations into adipose tissues. It is the key factor in the development of the type 2 diabetes and insulin resistance. The increase production of Chemokines such as C-C motif chemokine ligand 2, pro-inflammatory cytokines involving the interleukin 1β and 6, tumor necrosis factor α and also the reduced expression of the key insulin Adinopectin and Insulin sensitizing Adipokine. (Kratz, M.*et all* (2018).

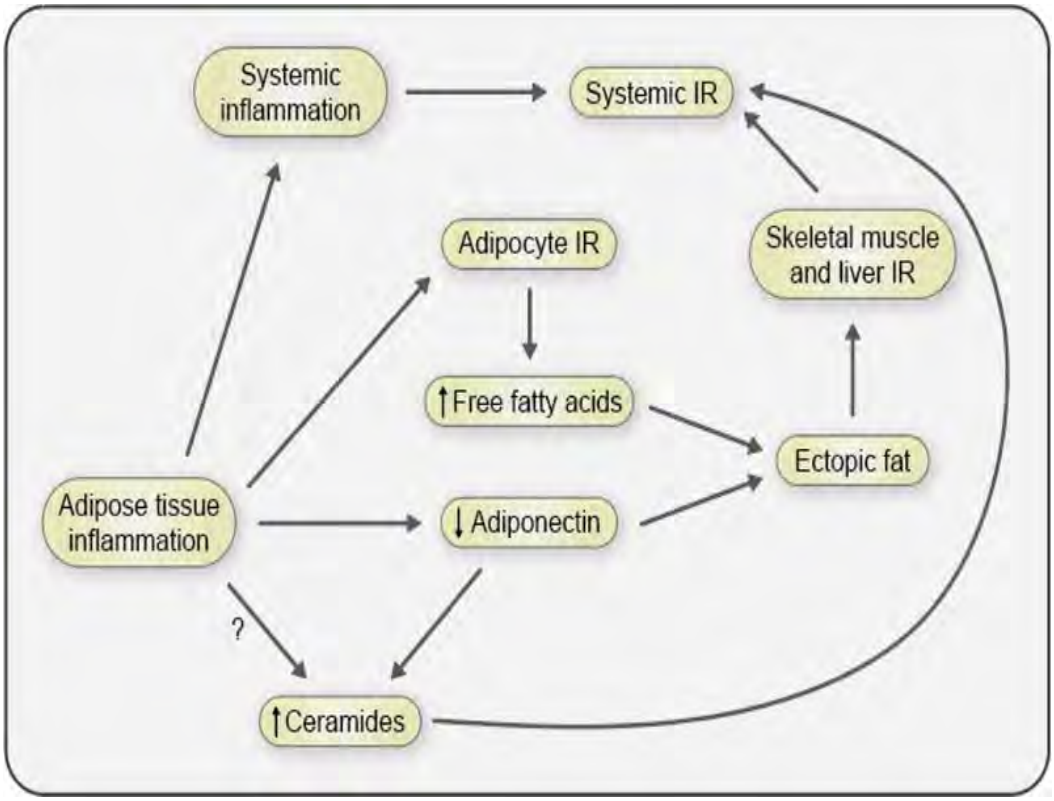

Figure1.4; Adipose tissue may be a stimulator in the development of systematic insulin resistance. (Kratz, M.*et all* (2018).

Obesity;

BMI > 30 kg/m2 is currently a widely accepted definition of obesity in adults. Although there are wide variations in obesity prevalence across developed market economies (such as Europe, the United States, Canada, Australia, etc.), a reasonable estimate points to an average prevalence of around 15-20%. These nations generally experience rising trends in prevalence over time. Although less prevalent in sub-Saharan Africa and Asia, where the majority of the world's population resides, obesity is nevertheless a problem throughout Latin America. But, there are rising rates of obesity there as well, and more crucially, rising rates of diabetes, particularly in Asian nations. At levels of BMI often regarded as acceptable in European and North American populations, the chances of type 2 diabetes mellitus tend to be high dramatically in these nations.

Changes in lifestyle frequently result in obesity and type 2 diabetes (increased sedentary lifestyles and increased energy density of diets). Both may be avoided by changing people's lifestyles at the population level, but doing so calls for a well-thought-out approach. Such plans are not created or put into action. These changes highlight the pressing need for international and national initiatives for the

management and prevention of type 2 diabetes and obesity (Seidell, J. C.2000)

Insulin resistance;

Insulin resistance is characterized by a weakened biological response to an increase or normal insulin level this indicates decreased sensitivity to insulin-mediated glucose elimination.

Mechanism;

The interactions of other hormones physiologically affect insulin's effects throughout the body. Although growth hormone and IGF-1 work in co-occurrence with insulin, which is the primary hormone regulating metabolic processes in the fed state, insulin produced growth Harmones among other stimuli, preventing insulin-induced hypoglycemia. The hormones glucagon, glucocorticoids, and Catecholamines are other counter-regulatory hormones. During a fast, metabolic activities are controlled by these Harmones.

Glucagon encouraged glycogenolysis, gluconeogenesis and ketogenesis. The Extend of phosphorylation or de-phosphorylation of the pertinent enzymes is examined by the ratio of insulin to glucagons. Lipolysis and glycogenolysis are promoted by catecholamines, while muscle catabolism, gluconeogenesis, and lipolysis are enhanced by glucocorticoids. Although excessive secretion of these hormones does not account for the vast majority of cases of insulin resistance, it may play a role in certain circumstances. (Wilcox, G.,(2005)

Hypertension or depression;

Diabetes and depression are on the rise in the United States, where 6.5% of adults have been diagnosed with the disease (1), type 2 diabetes is more common than type 1, and obesity is on the rise across the country. Around 16% of American adults will experience a depressive illness at some point in their lives, and this percentage rises when other types of depressive disorders are included, like dysthymia and mild depression. Hence, both researchers and policymakers should pay attention to the theory that depression and diabetes are causally linked.

Depression is associated with unhealthy lifestyle choices that raise the risk of type 2 diabetes, such as smoking, indulging in unhealthy foods, and not exercising (3). Moreover, central obesity and glucose intolerance are connected to depression.

Prevention;

It has been demonstrated that changes in lifestyle can postpone or stop the onset of type 2 diabetes. People should: in order to help prevent type 2 diabetes and its complications. To attain and keep a healthy body weight; be physically active by engaging in frequent, moderate-intensity activity for at least 30 minutes on most days. In order to control weight, more activity is needed;

Have a balanced diet and stay away from sugar and saturated fats. Smoking also raises your risk of diabetes and cardiovascular disease. (WHO)

CDKAL1 Gene;

The association of CDKAL 1 has been shown in various Ethnic groups. A significant association of the gene is with rs7756992, rs7754840, and rs10946398 in CDKAL1 with type 2 diabetes.

CDK5 regulatory subunit-associated protein is encoded by *CDKAL* 1 gene. The activity of CDK5 protein may be affected by this gene. As CDK5 show its affect in the process of insulin producing β cells of pancreas and stimulate insulin production. The degradation of β cells may results due to the enhance activity of CDK5 protein. And occurrence β cell dysfunction and predisposition to diabetes type 2 is thought to cause by CDK5.*CDKAL* 1 and CDK5 mediated pathways in β cells are thought to linked due to the expression of *CDKAL* 1 in human pancreatic islets. Impaired first phase insulin release is related to *CDKAL1* 1. (Huang, Q. Y.*et al* (2010).

Physiology of *CDKAL1*

CDKAL1 a tRNA modification enzyme, a mammalian methyl thio transferase that biosynthesizes 2-methylthio-N^6-threonylcarbamoyladenosine (ms^2t^6A) at position 37 of $tRNA^{Lys}$(UUU). When the rate of translation is high the ms^2t^6A modification in $tRNA^{Lys}$(UUU) is essential for its cognate codon misreading. In diabetes type 2 one of the most reproducible risk gene is CDKAL1 shown by a a number of whole genome associated studies. Insulin resistance are not associated to the changes in gene and obesity also not but with impaired insulin secretion. (Tomizawa, K.*et.,al* (2011).

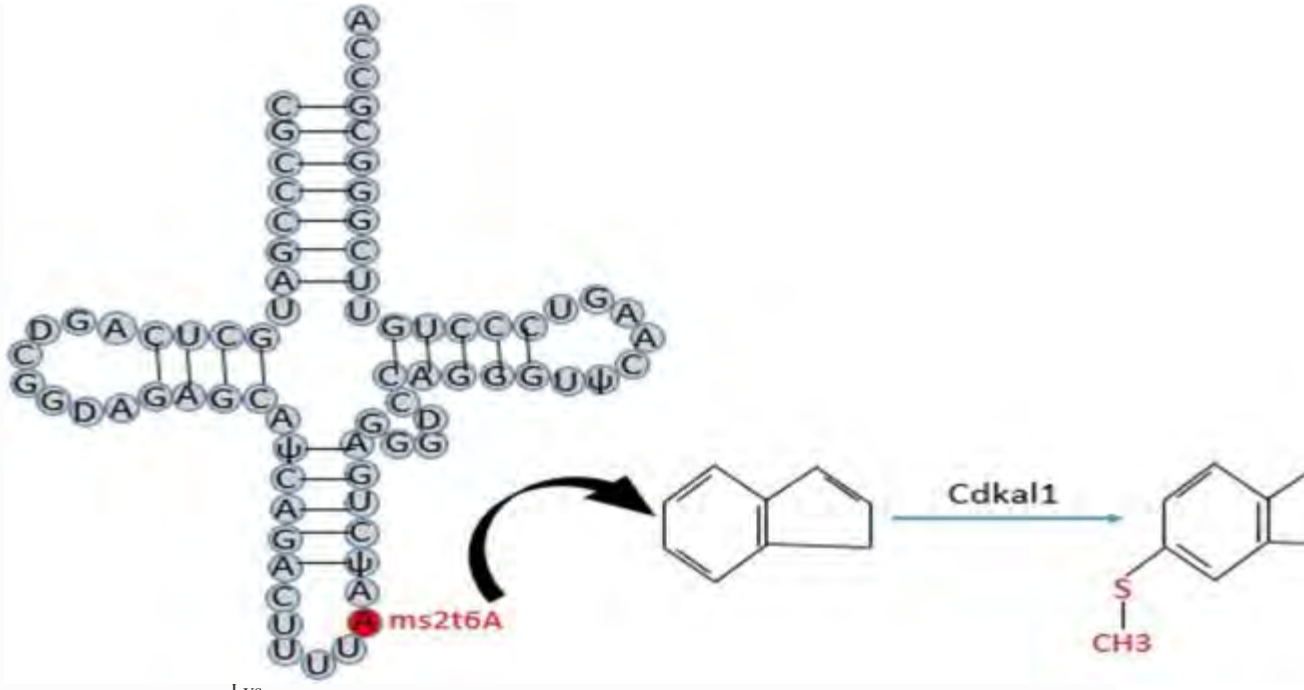

Figure 1.5 t-RNA^{Lys} modification by Cdkal1. The two Fe-S clusters present in Cdkal1 cause the addition of a 2-methylthio group (CH_3-S) at the 2C position of A37 residue of the t-RNA_{Lys}(UUU), which is necessary for the Lys amino acid recognition by the t-RNA(Roy, P *et a*l.,2022).

Treatment and diagnosis;

Diabetes should be managed with a nutritious diet and moderate exercise, according to general treatment guidelines. The major objective should be to decrease weight because type 2 diabetics are more prone to obesity. The calorie intake for each person should be based on his or her individual BMI and level of regular exercise. Protein intake should make up 10% to 20% of total calories ingested, whereas fat consumption should account for no more than 30% of total calories and no more than 10% of calories from saturated fat. The focus should be on consuming carbohydrates in general, regardless of the source, and avoiding the carbohydrates that absorb fat. Frequent exercise can help to lower the chance of developing diabetes. For type 2 patients, 30 minutes of moderate-intensity exercise each day can help lower blood pressure, shed pounds, and improve cholesterol levels.(Butt, S. M. (2022)

Drug therapy;

It is critical to provide the diabetic education to reach treatment goals. Nateglinide and repaglinide are the 2 fastest acting drugs used to treat diabetes. Metformin can also be added to treat hyperglycemia. Insulin therapy can also be helpful for the disease.

Prevention;

It is observed that lifestyle modifications can slow down the process of disease or it can prevent from T2DM. Therefore in order to prevent people should develop an active lifestyle. Do more exercise and intense activity for 30 minutes in order to control weight. Should take Nutritious and balanced diet. Avoid smoking as it greatly enhances the risk of cardiovascular diseases and diabetes. (WHO)

Aim;

To study the demographic and clinical characteristics of T2DM patients presented at THE DIABETES CENTRE ISLAMABAD.

Objectives;

1) Hospital approval for Patients Enrollment.
2) After the informed consent patients and controls were enrolled.
3) In a pre-designed questionnaire form all the demographic and clinical variables were recorded.
4) While considering the inclusion exclusion criteria blood samples were collected from both the patients and control.
5) To evaluate the relationship of CDKAL (rs10946398) gene polymorphism and its risk association among the T2DM patients Tetra ARMs PCR analysis was done.

Material and Methods

2.1 Study design

The purpose of study is to identify the important risk factors and a key role of CDKAL in diabetes type 2 patients represented at the THE DIABETES CENTRE Islamabad.

2.2 Ethical approval;

Study was approved by the supervisory committee at the department of zoology. For sample collection and data the ethical study was approved by MS TDC Islamabad. Patients were inducted through diabetic ward of TDC hospital Islamabad.

2.3 Sampling technique;

In epidemiological study, all the diabetic type 2 patients who visited were enrolled in study during September 2022 to December 2022 Patients details were noted on the designed Performa. Unique identification number was given to each patient on Performa. For about 3-5ml blood was taken from diabetic type 2 patients in EDTA coated tubes and was stored at -20

2.4 Questionnaire;

A questionnaire was designed to record all the information involving demographics, Patients medical and clinical history, past and current diabetic history and lifestyle. Patients were interviewed on their educational background, family history of type 2 diabetes to investigate the reported risk factors. It took almost 15 minutes to complete the Questionnaire. A written as well as verbal consent was taken from each **patient**.

2.5 INCLUSION Criteria;

Adults having age about 35 years or over, they are at risk for type 2 diabetes and should be tested at least every 3 years.

If you have risk factors that increase the onset of developing type 2 diabetes, you must be tested quite often and start regular screening earlier. Many of the risk factors involves family history of diabetes; and being overweight.

2.6 Exclusion criteria;

Young adults and Patients who were not willing to participate in the study were **excluded** from the criteria.

2.7 Risk factors involved in diabetes type 2

Suspected and established risk factors involved in diabetes type 2are given below;

Age;

Patients are at the age of 40 or above are at a high risk of diabetes type 2 therefore the age of controls and patients was noted in the questionnaire.

Family History ;

Family history also plays an important role on the onset of diabetes mellitus type 2. Patients were asked if they have there any first degree (father, mother, siblings) or second degree relatives (grandmother ,aunt) were diagnosed with Diabetes Mellitus type 2. Family history was recorded in the questionnaire.

Insulin resistance;

Insulin resistance can promote a person's risk for developing impaired glucose tolerance and type 2 diabetes. Insulin resistance in individual share many of the same risk factors as those are involved with type 2 diabetes. This includes Hyperinsulinemia, Atherogenic dyslipidemia, glucose intolerance, hypertension, prothrombic state, hyperuricemia, and polycystic ovary syndrome.

Obesity;

Although not all the individuals who are obese develop type 2 diabetes and not all with type 2 diabetes are obese [3]], the current increase in the pace of obesity has been linked with an increase in the prevalence of type 2 diabetes [[4]]. In the past, type 2 diabetes has mainly affected middle-aged individuals [[3]. Thus, it is especially dangerous that the increasing prevalence of obesity in the US pediatric population has been combined by an increase in prevalence of type 2 diabetes among children and adolescents [[6]]. Therefore Patients lipid profile was also recorded.

Clinical characteristics of Type 2 Diabetes;

Patients those were positive of diabetes type 2 were asked for their medication, whether they were following a special diet or any treatment measures. Lipid profile was recorded for 30 patients. Creatinine and HBA1C were also recorded for 100 patients. All the possible data was obtained and noted from patients.

Compilation of patient's data risk factors analysis;

All the data collected for different variables were organized using Microsoft excel

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REAGENT PREPARATION FOR DNA EXTRACTION
SOLUTION A-LYSIS SOLUTION

Constituents of solution A are 0.32 M sucrose, 10Mm Tris (PH=7.5),5mM $MgCL_2$ and Triton X 100(1% V/V)

SOLUTION B

Constituents of solution B are 400mM NACL, 10Mm TRIS (pH =7.5) 2Mm EDTA (PH 8.0)

SOLUTION C

Solution C in DNA extraction is referred as phenol which helps to remove non-polar proteins and help to remove lipids from solution.

SOLUTION D

Solution D constituents involves chloroform and Isoamyl Alchol in ratio of 24;1 chloroform removed DNA from phenol. It can also help to removes proteins.by using phenol chloroform method Iso-amyl alcohol removes foaming that result at the time of DNA extraction. Ratio disturbance in any proportion of chloroform phenol results in extraction of other constituents such as RNA.

Proteinase k

Working solution for about 100mg/ml was used.

20% SDS

In 50mL water 10g sodium dodecyl sulphate.

70% Ethanol

60ml distilled water was added 140 absolute ethanol (molecular grade) to prepare 70% ethanol.

2mM TE Buffer;

1Mm EDTA, 10Mm TRIS hydroxyl (methyl amino) methane,(PH 8.0)

DNA extraction from blood samples;

Blood sample about 2ml from each patient was collected in EDTA coated tubes and the blood samples were stored at -20 *c until the DNA was extracted from the phenol chloroform extraction method. It took about three days to complete DNA extraction.

DAY 1;

- At room temperature blood samples were thawed for Red blood cell (RBC) lyses.
- An EPPENDORF for about 1.5ml labelled with the UAI number of the patient and then 700µl of blood and 700µ of solution A poured into it. Thoroughly mix the blood and solution and kept at room temperature for about 20 minutes for appropriate lysis.
- Centrifugation was done at 13000 rpm for 10 minutes.
- Half of the supernatant was discarded properly in a discarder box containing bleach to avoid any contamination.
- In 400µl of solution A nuclear pellet was dissolved and again centrifuged at 13000RPM.
- Now supernatant was completely discarded and in solution A again dissolve the nuclear pellet.
- Again centrifuged at 13000 for 10 minutes, supernatant was discarded by shaking it vigorously that was obtained in 400µl of solution B, 25µl of 20% SDS and 12µl of proteinase K.
- At 37*c the EPPENDORF tubes containing samples were incubated for the digestion of proteins in the pellet of WBCs.

DAY 2;

- A fresh mixture of solution C and D was prepared on the next day by taking equal volume of both solutions (50:50) A freshly prepared solution C and D was added to each sample and centrifuged at 13000 RPM for 13 minutes**.**
- Two layers were formed because of centrifugation supernatant was shift to a new labelled EPPENDORF.
- To the separated layer add solution D for about 500µ shake thoroughly and at 13000 RPM centrifuge for 10 minutes. The upper layer was transferred to a new labelled EPPENDORF tubes.
- Chilled sodium acetate for about 55µl (3M, PH 5-6) and chilled iso-propanol for about 500µl were added.to precipitate genomic DNA sample was overturned several times.

- Then the sample was centrifuged at 13000 RPM for 10 minutes pellet was form as a result..
- Supernatant was discarded; 200µl of 70% ethanol was added for washing purpose and than centrifuge at 13000rpm for 10 minutes.
- Supernatant was discarded again and in VACCUM concentrator pellet was allowed to dry.
- A suitable volume (80-200µl)of TE buffer added after the DNA was air dried and incubate overnight at 37*C

Day 3;

On third day extracted genomic DNA having volume of about 5µl was mixed with 5µl of bromophenol blue dye allow to run on 1% agarose gel for qualitative and quantitative analysis. UV Transilliuminator (Biometra ,Gottingen, Germany) analyzed the results gel doc system and stored at -20 c. Denaturation of extracted DNA was avoided by giving heat shock in water bath at 37*c for 1 hour.

Quantification And Purity Assesment Of Dna
For determining the concentration of DNA two methods were used;
Agarose gel electrophoresis estimation with a known standard DNA Dilution;

UV-induced florescence of Ethidium bromide dye can be used intercalated the dye into nucleic acid. The amount of nucleic acid and the amount of fluorescence are proportional to each other. Comparison of the fluorescence from the test DNA and from a known amount of a DNA was done also helps in the rapid assessment of the integrity of the nucleic acid. The DNA was stored at -20*c.

Single nucleotide polymorphism;

A single nucleotide polymorphism is the most basic type of polymorphism in human genome consisting of any insertion, replacement, deletion. In genome these SNPs can be found in both the coding and non-coding region and can be utilized to diagnosed disorder.

TETRA ARMS PCR;

The tetra-primer amplification refractory mutation system–polymerase chain (ARMS–PCR) reaction is a low cost and easy method to for SNPs genotyping. Four primers can be used in a single PCR after which the gel electrophoresis can be done.

The use of tetra-primer ARMS–PCR meet the standards of modern genomic research and allows in a fast, reliable and low cost way of the study of SNPs.

In a single PCR 4 primers were used by the tetra –primer ARMS-PCR to determine the genotype. Two non-allele-specific primers in the beginning amplify the region that involves the SNP. And these are called as outer primer. The outer primer fragment serves as a template to the two allele-specific primers (inner primers) which will produce the allele-specific fragments [21]. The two allele-specific fragments can be distinguished by their different sizes in an agarose gel [by placing outer primers at different distances from the polymorphic nucleotide.

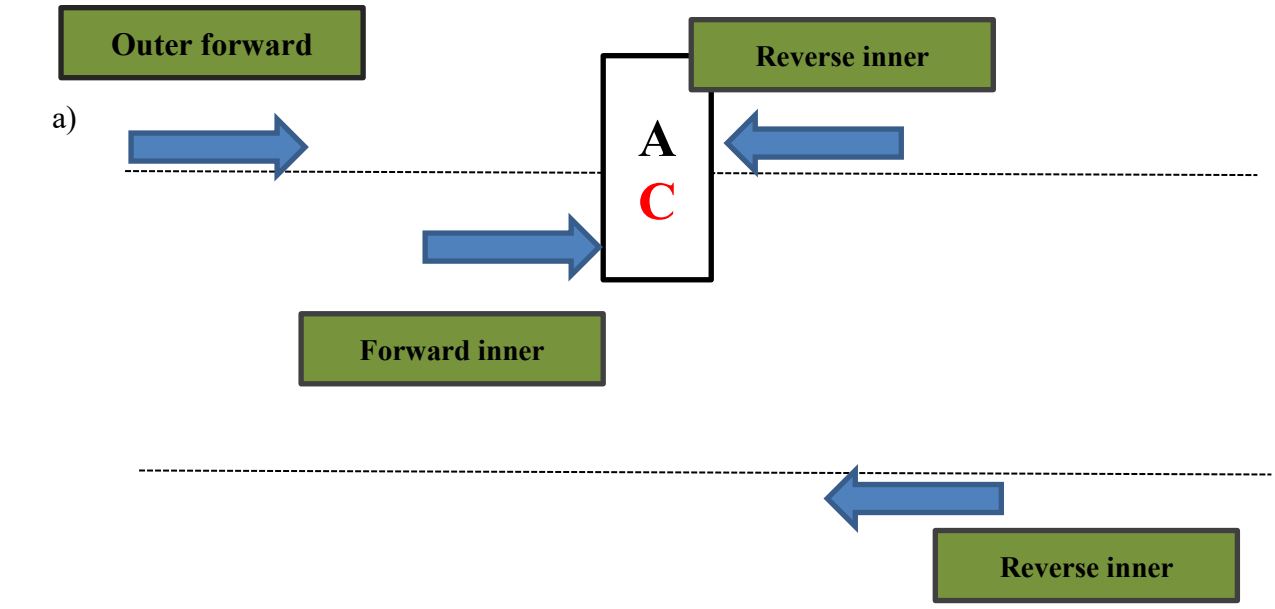
Optimization of T-ARMS PCR must be done for the successful completion of the procedure as it allows to prevent the non-specific bands. The ratio of outer to inner primer must be appropriate for PCR also to identify the correct annealing temperature would be the measure to improved optimization.

The PCR product was used to separate by 2% Agarose gel and the gel visualized using gel documentation system.

Primer designing;

Manually designed the four primers (forward and reverse) for the CDKAL gene (rs2853669) using the promoter region sequence of the CDKAL gene, the size of the fragments were limited to 100-650bp.primer 3 plus software was used .Using the NCB blast software(https://www.ncbi.nih.gov/tools/primer-blast/) and in-silico PCR tool UCSC genome browser (http://genome.ucsc.edu/cgi-binhgPcr),primer'specificity was assessed. The properties of each primer checked by PCR primer stats sequence manipulation suite. Sequence of the primer with their product sizes and melting temperature have been listed in

The FASTA sequence of CDKAL1 rs10946398 was downloaded from NCBI BLAST for primer designing. Reported alteration was detected by mutation taster. Interested source sequence was copied and pasted in primer 3 plus, after all the parameters were settled like number of base pair GC content, primer size and then select the pick primer. Specific primer outputs we get. Annealing temperature and primers specificity was checked by primer stats software.



b)

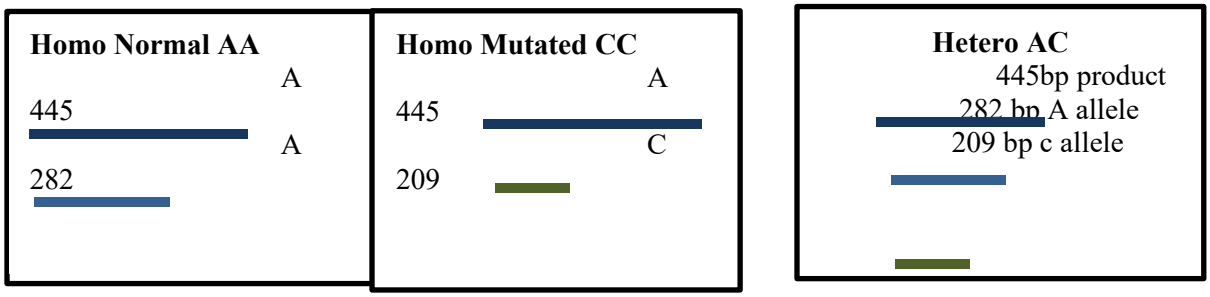


Figure 2.1 (a) Binding position of outer and inner forward reverse primers. (b) Expected bands obtained with sizes of 445bp, 282bp, 209bp of all the possible genotypes AA,AC, and CC genotypes. PCR Amplifications;

PCR amplifications can be perform by taking 0.2ml PCR tubes which were utilized to carry 25µl total volume. Each tube contain;

- 2.5µl of genomic DNA (40-50ng/1)
- 2.5µl of Taq buffer,
- 2.5µl of MgCl2 (25Mm)
- 0.5µl of de oxy nucleotide triphosphate (dNTPs) mix(2.5Mm) each.
- 6µl of forward and reverse primers (both internal)
- 0.5µl of reverse and forward outer primers (10mM)

- 1µl of Taq polymerase enzyme

14µl of autoclaved and distilled water (DNASE/RNASE free)

The tubes were centrifuge at 13000rpm for 30 sec for thorough mixing. In a thermal cycler(-) the PCR amplification was done at 95*c for 5 minutes, followed by 35 cycles of 95*c for 1 minute of denaturation, temperature about 54-64*c for 45 seconds for annealing,72*c for 30 seconds (extension), and for final extension at 72*c for 10 minutes. A touchdown PCR method for proper annealing with a decrement of 1*c for ten cycles. Each tube contain a volume of about 25µl.

Visualization of the amplified product;

The Amplicons were loaded on a 2% Agarose gel with PCR product of about 2.5µl and loading dye about 2.5µ. DNA ladder of 100 Bp having volume about 2.5µwas also loaded in one of the wells. The sized of the bands which emerged on the gel were compared to the sizes in the ladder when viewed with the transilluminator under UV light, and the gel photos were saved in the gel documentation system.

Table 2.1 Primer sequence of CDKAL 1 Gene

CDKL primer ID	**Primer sequence**	**Tm bp**
CDKL-F-Inner	GGAAAAGGGTTTAGTATCGCTC	58 22
CDKL-F-OUTER	CTTGGAGTAGTCACCTGGTCAT	58 22
CDKAL-R-INNER-A	GATGACTTGATGCAATGACAGTAT	58 24

CDKL-R-Outer GCAAGCAGTTGATTTTTTTTTC 58
22

Tetra –ARMS PCR Product size information for CDKAL1 rs10946398

Primer	System	Allele	Amplicon
CDKAL F1	Forward inner primer	C	209
CDKAL R1	Reverse inner primer	A	282
CDKAL FO	Forward outer primer		445
CDKAL RO	Reverse outer primer		

Statistical Analysis;

DNA samples are visualized on gel after performing the TETRA ARMs PCR and alleles were recorded for each sample in table 2.1. Percentages and frequencies were used to represent the data. For statistical analysis SPSS Statistics 21 was used. Allele and genotypes were collected by direct counting. P value was 0.25 as statistical analysis.

Results

This study consists of 2 parts. First involves the risk factor analysis using demographic clinical characteristics of Diabetes type 2 patients at THE DIABETES CENTRE ISLAMABAD. Second part is to check the *CDKAL 1* r s 10946398 polymorphism in T2DM.During our period of study we have collected the clinical data from 100 patients**.**

3.1 Analysis of baseline characteristics of the T2DM patients;

The demographic characteristics of the patients including their age, BMI, HBA1C values, creatinine values and the patients lipid profile was recorded on Excel. And these recorded baseline characteristics of patients were represented in frequency and percentage using SPSS software. Starting from the age, there was a vast range of ages that patients were belong to so for convenience the data was divided in to groups starting from 27-37 to 70 or above and it was found that most of the patients were lie in 49-59 and 38-48 age group with a percentage of 34% and 29% respectively.

The HBA1C values of patients were also recorded. HBA1C refers to the plasma glucose over the past 8 to 12 weeks and is the screening test for T2DM.For diagnoses an HBA1C of 6.5% is recommended as the cut point but the HBA1Cs less than 6.5 does not exclude disease diagnoses. In our data most of the patients have their HBA1Cs below or equal to 6 were 6% while rest of 94 patients had higher HBA1Cs values.

Patient creatinine values were recorded from the hospital laboratory. Normal range for serum Creatinine is 0.8- 1.4mg/dl. It has been observed that females usually have lower creatinine values than males because they have less muscle mass. In our data patients had creatinine values equal or less than 1.5 were 91% while 6% of them had more than 1.5mg/dl creatinine.

Obesity also plays a significant role in diabetes therefore out of 100 Patients lipid profile of 30 patients was also recorded from laboratory which includes the body cholesterol and triglycerides levels.

Normal cholesterol in adults should be less than 200mg/dl. In T2DM patients 19% of the patients have cholesterol level lies in between 100-200mg/dl. Out of 30 patients 6 of them had cholesterol less than 115mg/dl while 24 of them had higher cholesterol levels.T2DM Patients **Triglyceride levels** recorded as well. Having high triglyceride

levels may be a sign that you may have diabetes. The normal level of triglycerides should be less than 150mg/dl. In present clinical data most of the patients had higher triglyceride levels more than 150mg/dl which comprises about 23% while 7% had lower than 150mg/dl.

Table 3.1 Clinical Characteristics of T2DM Patients

	Groups	Frequency	Percentage%
AGE	≤35	5	5%
	35-45	23	23%
	45-55	28	28%
	55-65	24	24%
	>70	5	5%
Gender	Male	54	54%
	Female	46	46%
Cholesterol	<150	6	20%
	>150	24	80%
Creatinine	≥1.5	6	6.18%
	≤1.5	91	93.81%

Triglycerides	≤150	7	23.3%
	≥150	23	76.6%
HBA1C	≤6	6	6%
	≥6	94	94%

3.2 Analysis of genotype and allele frequency of *CDKAL1* variants rs10946398 in patients and Controls

The variant rs10946398 for CDKAL1 gene, the wild type is A and C is mutated and the minor allele C is reported. Upon genotyping data shows that 47.5% are homozygous AA, 41.1% are heterozygous CC and about 11.7% are homozygous CC. For controls, data revealed that among them 60% have homozygous AA genotype, 30% have AC genotype while if we check the allele C its only 10%. However the allele A is about 47.5% in cases and 60% in controls.

Table 3.2 Frequency and percentage of genotypes and their alleles in cases and controls.

z	Cases		Control		P value
AA	8	47.5%	6	60%	
AC	7	41.1%	3	30%	

CC	2	11.7%	1	10%	0.25

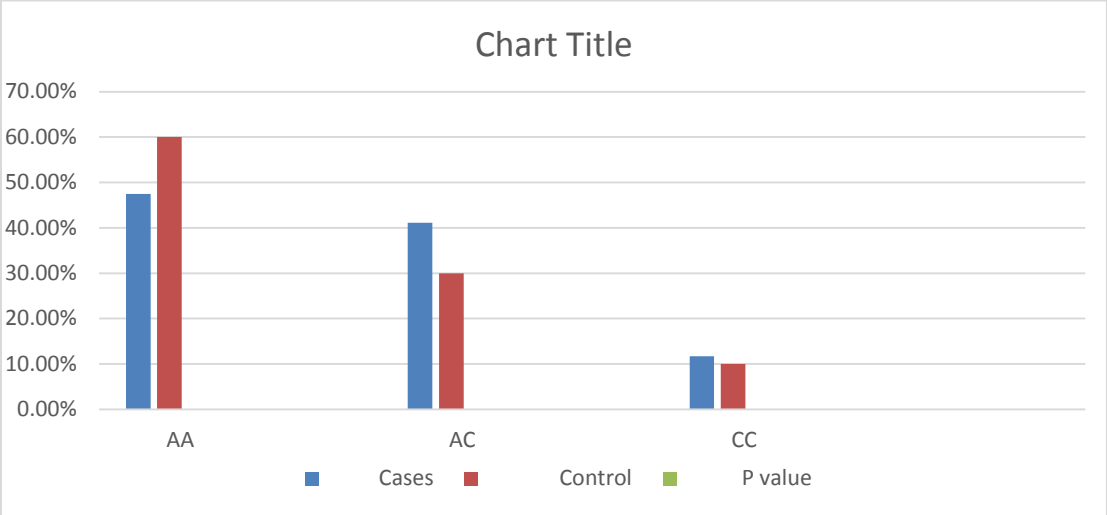


Figure 3.1 Bar chart showing the A and C allele frequency in patients and control

3.3 Molecular analysis of cases and control;

The present case control study was conducted to check the potential association of CDKAL1 gene promoter variant (rs10946398) in 17 T2DM patients and with 10 controls. After the experimentation involving, DNA extraction, primer designing, tetra ARMs PCR and gel electrophoresis amplification alleles were counted and listed in table 3.1.Obtained on 2% gel the genotypes were called based on fragment size. The genotypes were homozygous AA, homozygous CC, and heterozygous AC.

Numerous variables which might affect the efficiency of PCR involving buffer volume, primer concentrations MgCL2 and PCR cycling settings were optimized. Annealing of the temperature ranges from 64-54 for better results of PCR.

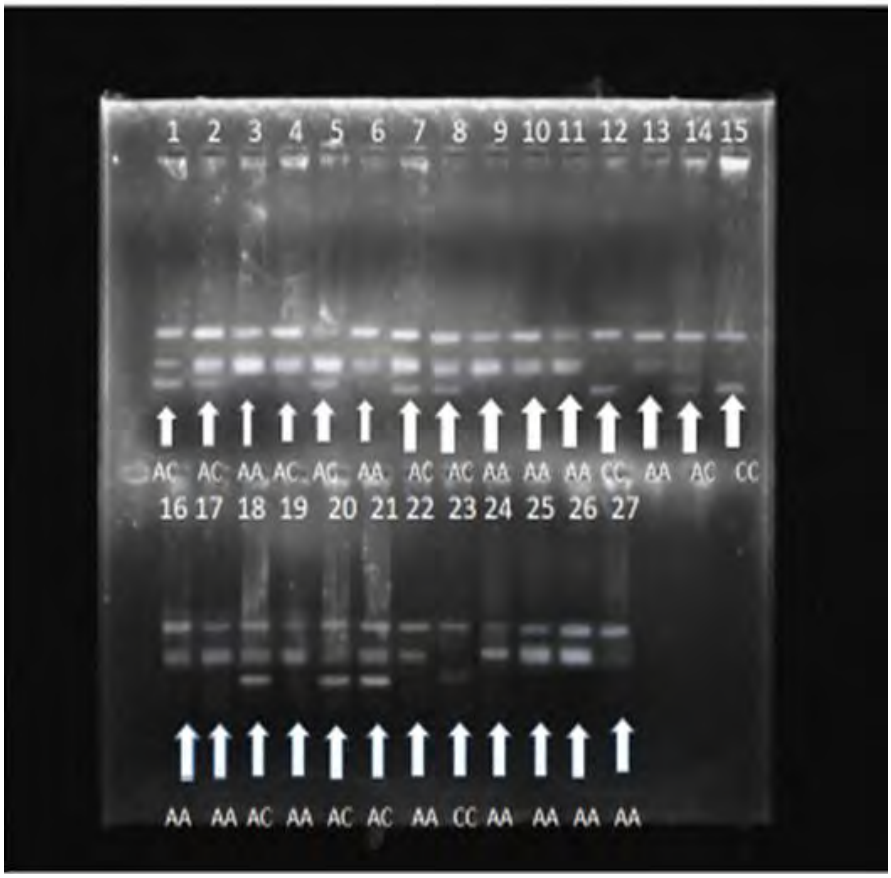


Figure 3.2 A 2% Agarose gel showing the PCR products of CDKAL1 region polymorphism rs10946398.PCR Amplified products following the genotype homozygous AA appear in lane 3,6,9,10,11,13,16,17 for patients and in 19,22,24,25,26 and 27 for control.. The genotype heterozygous AC appear in lane 1,2,4,5,7,8 and 14 for patients while in 18,20,21 for control. And the homozygous CC appear in lane 12 and 15 for patients while in 23 is for control.

Table 3.3 Revealed genotypes of cases and controls

Cases ID	Band sizes 445bp 282bp 209bp	Genotype	Control Id	Band sizes 445bp 282bp 209bp	Genotype
T2DM**1**	✔ ✔ ✔	AC	C1	✔ ✔ ✔	AC
T2DM**2**	✔ ✔ ✔	AC	C2	✔ ✔	AA
T2DM**3**	✔ ✔	AA	C3	✔ ✔ ✔	AC
T2DM**4**	✔ ✔	AA	C4	✔ ✔ ✔	AC
T2DM**5**	✔ ✔ ✔	AC	C5	✔ ✔	AA
T2DM**6**	✔ ✔	AA	C6	✔ ✔	CC
T2DM**7**	✔ ✔ ✔	AC	C7	✔ ✔	AA
T2DM**8**	✔ ✔ ✔	AC	C8	✔ ✔	AA
T2DM**9**	✔ ✔	AA	C9	✔ ✔	AA
T2DM**10**	✔ ✔	AA	C10	✔ ✔	AA

Discussion;

Diabetes mellitus type 2 is a globally prevalent disease. It comprises more than 90% of all three type of diabetes. Type 2 diabetes results in insulin deficiency caused by the pancreatic β cell dysfunction and insulin resistance. The disease epidemiology is affected by both the environmental and genetic factors. Environmental factors include excessive fat and sugar consumption, lack of physical activity.(Davies, M. J *et al*.2017).

Type 2 diabetes is often more related to age of onset of disease is usually over 35 however cases exists in lower age as well. The high risk countries for diabetes type 2 reported are India and china. (Groop, L.*et al* 2015). The current prevalence of diabetes in Pakistan is 11.77%.Prevalence in males is higher than the females and is about 11.20% in males and 9.19% in females. (Arain, S. A. *et al* 2016).

Different factors contribute to the pathogenicity of diabetes type 2 these include genetic and non genetic factors as well. Non genetic more often related to age, lack of physical activity, lifestyle, organic pollutants ,food and its components linked directly or indirectly to oxidative stress and obesity which than result in the onset of diabetes .Although non genetic factors play a major role on the onset of diabetes however genetic factors may also involved in the pathogenesis. Inspite of advanced genetic tools the genetic variants which increases the risk of diabetes type 2 are only 10-30% have been identified.(Liu, W.*et al* 2019)

In our study we collected data based on the risk factors for the disease. Data regarding age, gender, HBA1C was recorded for 100 patients whereas lipid profile was only available for 30 patients.

The age distributions of T2DM patients were lies in between 45-55 is 28% and from group between 55- 65 is %. Our study matches with the study of(Groop, L.*et al* 2015). According to which usually the onset of the disease is over 35 years.

In present study the number of the diseased cases were more in males than in females. 54% of the males had type 2 diabetes mellitus while 46% cases of the females were recorded. Previous study reported that the occurrence of disease was more in males than in females. Arain, S. A. 2016. D. (2019)

Obesity and type 2 diabetes are linked side by side. About 60 t0 90 % of the diabetic patients have been obese. Ybarra, J. (2005). Obesity is not only a risk factor

but also causes diabetes. In present study we have collected the data of Patients lipid profile which includes the cholesterol and triglycerides levels.

Cellular cholesterol accumulation might leads to β cell dysfunction. Diabetic patients shows a number of lipid abnormalities associated with cholesterol and fatty acid excess.in recorded data more of the patients had higher cholesterol levels about 80% lies above 150mg/dl and while 20% of the patients had lower than 150mg/dl. Previous study showed that higher cholesterol levels may results in β cell dysfunction which leads to diabetes. (Norata, G. D.*et al* 2019)

For Diabetes type 2 the higher triglyceride levels are common dyslipidimic feature. A fasting triglyceride level > 150mg/dl is one of the main criteria for defining the individuals which are at higher risk of the disease.

There is a linear relationship between the triglycerides and T2DM. Higher triglyceride levels results in hazardous ratio for the presence of diabetes type 2. The association between this occurrence is independent of sex, age, hypertension and BMI .In present study the data we collected patients reports which showed higher triglyceride levels about 76.6% had higher values than 150mg/dl and 23.3% had less than 150mg/dl. Our study results matches with the study of (D. *et., al* 2019)

The main goal of the present case control study was to check the association of *CDKAL1* single nucleotide polymorphism (rs10946398) with type 2 diabetes in Pakistani patients. In Pakistan no such study was conducted before to investigate the association of *CDKAL1* variant (10946398) with type 2 diabetes mellitus. In the pathogenesis of T2DM the C allele (r s 10946398) has been reported in different populations. Here some research confirmed this association by reporting this association in different population.

Four SNPs in *CDKAL1* (rs4712523, rs10946398, rs7754840, and rs7756992) reported to associated with type 2 diabetes in Pima India.

Another study identified SNP rs10946398 (*CDKAL1*) in type 2 diabetes mellitus complications in Chinese Han population. Lin, X. *et*., 2008).

In this study the distribution of *CDKAL1* variant (rs10946398) the genotype (AA, AC, CC) in type 2 diabetic patients (47.5%, 41.1%, 11.7%) were different from controls (60%, 30%, 10%) The incidence of C allele in CC genotype is more in cases than in control.

In conclusion this is the first study of screening of rs10946398 *CDKAL1* polymorphism in Pakistani population, patients (17) and (10) controls. And there is non-significant association of the disease has been observed. The findings of the study does not support the idea of association of *CDKAL*1 gene variant rs10946398 in Pakistani population.

However this is a small scale study. The findings could be improved by increasing the sample size for genotyping and a large scale cohort studies are required to elucidate the association of rs10946398 in Pakistani population, genetic studies should be carried out to find the other variants of *CDKAL* 1 as well as other diabetes mellitus type 2 risk genes in Pakistani population.

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