Association of Genetic Variation in TCF7L2, SLC22A1 and KCNJ11 Genes with Risk for Type 2 Diabetes in Bangladeshi Population

Ph.D THESIS

A Dissertation Submitted to the University of Dhaka in Partial Fulfillment of the Requirement for PhD degree in Biochemistry and Molecular Biology

Submitted by-

Registration no: 96
Session: 2016-2017
Department of Biochemistry and Molecular Biology
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To Whom It May Concern

I hereby declare, in accordance with the by-law of the University of Dhaka, that the thesis work entitled "**Association of Genetic Variation in TCF7L2, SLC22A1 and KCNJ11 Genes with Risk for Type 2 Diabetes in Bangladeshi Population"** describe here is entirely own of Amrita Bhowmik. This work was conducted and carried out under my supervision while she enrolled in the degree of doctorate of philosophy in Biochemistry and Molecular Biology in the faculty of Biological Science at the University of Dhaka. All information in this document has been obtained and presented in accordance with academic rules and ethical conduct and have been recommended for the award of PhD of Science. To the best of our knowledge no part of the research work has been submitted for other degree or qualification in an institute at home or abroad.

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Dedicated to-

My Beloved Family

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The Author

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List of abbreviations

Name	Details
ABCC8	ATP-binding cassette transporter subfamily C member 8
ABF	After breakfast
ADA	American Diabetes Association
ADAMTS9	ADAM metallopeptidase with thrombospondin type 1motif,9
ADCY5	Adenylate cyclase 5
ADPKD	Autosomal Dominant Polycystic Kidney Disease
ALT	Alanine amino transferase
ANOVA	Analysis of variance
Aps	Action potentials
ATP	Adenosine triphosphate.
BCL11A	B-cell lymphoma/ leukemia 11A
BIHSH	Bangladesh Institute of Health Sciences hospital
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes Endocrine and Metabolic Disorders
BTED5	Basic transcription element binding protein
BMI	Body mass indexes
C2CD4A/BC2	Calcium-dependent domain containing 4B
cAMP	Cyclic adenosine monophosphate
CDC123	CAMK1D Cell division cycle 123
CDKAL1	CDK5 regulatory subunit associated protein 1-like 1
CDKN2A/B	Cyclin-dependent kinase inhibitor 2A and 2B
CE	Cholesterol esters
CETP	Cholesteryl ester transfer protein
COD-PAP	Cholesterol Oxidase/ Peroxidase
DBP	Diastolic blood pressure
DGKB/TMEM195	Diacylglycerol kinase beta and Transmembrane protein 195
DIAGRAM	Diabetes Genetics Replication And Meta-analysis
dLDL	Small, dense LDL
DZ	Dizygotic
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FTO	Fat mass and obesity associated
GAD65	Glutamic acid decarboxylase-65
GCK	Glucokinase
GCKR	Glucokinase regulator
GDM	Gestational diabetes mellitus
GINR	Glucose insulin Ratio
GOD-PAP	Glucose oxidase- Peroxidase

GWAS	Genome wide association scans
HbA_{1c}	Glycosylated Heamoglobin
HDL	High Density Lipoprotein
HDLn	Nascent HDL
HHEX	Hematopoietically expressed homeobox
HL	Hepatic lipase
HNF1B	Hepatocyte nuclear factor-1β
HNF4	Hepatocyte nuclear factor-4
HOMAB%	Beta cell function
HOMA-IR	Insulin Resistance
HOMAS%	Insulin Sensitivity
HPLC	High Performance Liquid Chromatographic
HSL	Hormone- sensitive lipase
IDF	International Diabetes Federation
IGF2BP2	IGF-2 mRNA binding protein 2
IGT	Impaired glucose tolerance
IRS1	Insulin receptor substrate
JAZF1	Juxtaposed with another zinc finger gene 1
KCNJ11	Potassium inwardly-rectifying channel, subfamily J, member 11
KCNQ1	Potassium voltage-gated channel, KQT-like subfamily, member 1
Kir6.2	Inward-rectifier potassium ion channel
KLF14	Kroppel like factor 14
LADA	Latent adult-onset autoimmune diabetes
LDC	Least developed country
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
MAF	Minor allele frequency
MODY	Maturity-onset diabetes of the young
MTNR1B	Melatonin receptor 1B
MZ	Monozygotic
NBD1	Nucleotide-binding domain 1
NBD2	Nucleotide-binding domain 2
NOTCH2	Notch homolog 2
OCT-1	Organic cation transporter-1
PCR	Polymerase chain reaction
PNDM	Permanent neonatal diabetes mellitus
PPARG	Peroxisome proliferator-activated receptor γ
PROX1	Prospero protein homeobox 1
QUICKI	Quantitative Insulin Sensitivity Check Index
RFLP	Restriction fragment length polymorphism

RRP	Readily releasable pool
SBP	Systolic blood pressure
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SGs	Secretory granules
SLC22A1	Solute carrier family 22, member 8
SLC30A8	Solute carrier family 30 (zinc transporter), member 8
sLDL-R	LDL receptor
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
SR-B1	Scavenger receptor B1
SUR1	Sulfonylurea receptor 1
T1DM	Type 1 diabetes Mellitus
T2DM	Type 2 diabetes Mellitus
TBF	Total body fat
TCF7L2	Transcription factor 7-like 2
TG	Triglyceride
THADA	Thyroid adenoma associated
TNDM	Transient neonatal diabetes mellitus
TSPAN8-LGR5	Tetraspanin 8 and Leucine-rich repeat- containing G protein- coupled receptor 5
UBE2E2	Ubiquitin conjugating enzyme E2F2
UTR	Untranslated region
WFS1	Wolfram syndrome 1
WHO	World Health Organization
WHR	Waist Hip ratio

Abstract

Type 2 diabetes mellitus (T2DM) is a multifactorial disease. Its prevalence has been rapidly increasing throughout the world, which is associated with several gene polymorphisms including KCNJ11, TCF7L2, SLC22A1 etc. The aims of this study were designated to investigate the association of KCNJ11 (rs5219), TCF7L2 (rs12255372) and SLC22A1 (rs628031) gene polymorphisms with T2DM in population of Bangladesh.

In a case-control study with 697 unrelated subjects, 326 healthy controls and 371 diabetic patients (diagnosed based on American Diabetes Association criteria) were recruited for this study. The serum fasting glucose, lipid profiles, creatinine, alanine amino transferase (ALT), HbA_{1C} and serum insulin level were measured by standard methods. HOMA B%, HOMA S% and HOMA IR were calculated by HOMA-SIGMA software version 2.2. QUICKI and Secretory HOMA were calculated by standard formula. Chemical method was used for DNA extraction from whole blood sample. PCR-RFLP method was used to detect KCNJ11 E23K (with Ban II), TCF7L2 (with Nla III) and SLC22A1 gene polymorphism (with MSc I) by restriction enzyme digestion. Data were analyzed using independent *t*-test, Chi-square or Fisher exact test, as appropriate. $p<0.05$ was considered significant.

Baseline data showed that FBG and HbA1c% level of diabetic group was significantly higher than control group ($p<0.001$). HOMAB% ($p<0.001$); HOMA S% ($p<0.001$), QUICKY ($p<0.001$) and secretory HOMA ($p<0.005$) were significantly lower; whereas insulin resistance was significantly higher ($p<0.001$) in diabetic subjects compared to control respectively. The TG and LDL levels were significantly higher ($p<0.001$) in diabetes group compare to control group respectively.

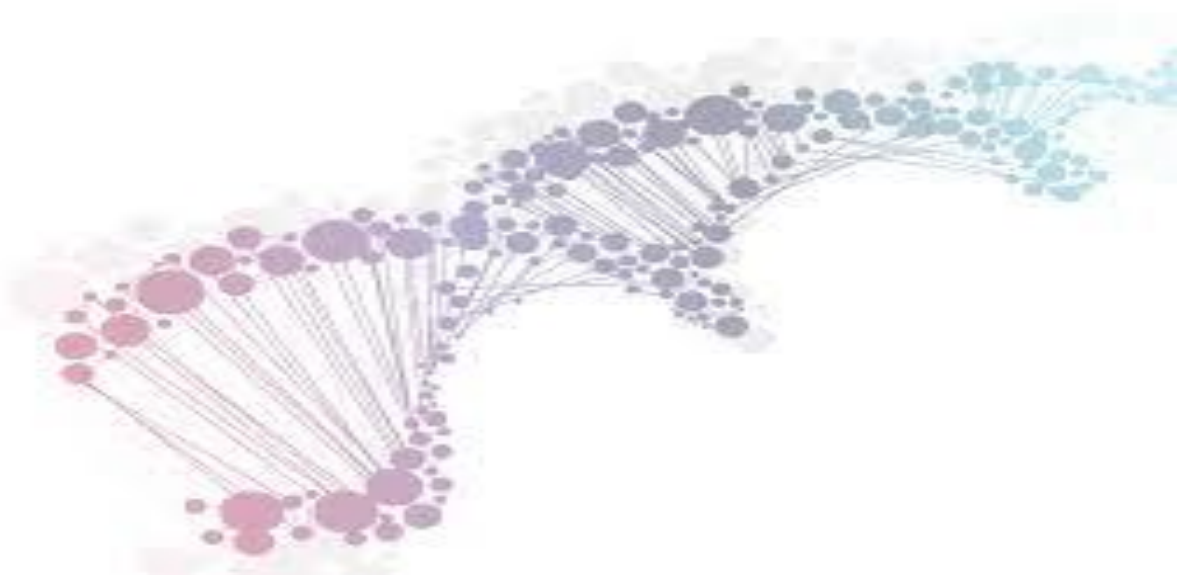
Statistically significant associations exist among E23K, K23K and E23K+K23K variants of KCNJ11 gene with T2DM where the E23E genotype was considered as reference group; and K allele of cases were significantly higher than E allele ($p<0.005$). E23K variant has high risk for predisposition of diabetes in both male and female respectively. The frequency of E23K variant was significantly higher in diabetic subjects with positive family history of diabetes compared to without family history; and also showed a diabetic risk factor for semi urban and urban population than rural subjects. No association was found among genotypes with diabetogenic parameters.

TCF7L2 genome analysis was revealed a significant association of TT and GT+TT variants in diabetic group, when the GG genotype was considered as reference group. TT variants have high risk for predisposition of diabetes in male than female; and with positive family history of diabetes than without family history. GT and TT variants were shown a significant association with semi urban and urban diabetic subjects than rural. In relation to diabetogenic parameters, a significant increase of QUICKY was found in TT variants in diabetic group ($p<0.05$) compared to GG and GT genotype. Whereas, HOMA IR ($p<0.05$) and QUICKY ($0<0.001$) were significantly higher in GT+TT variants than GG variant respectively.

The risk for T2DM was also calculated in relation to SLC22A1 gene. There was significant relationship exist among GA, AA and GA+AA variants when GG genotype was considered as reference group respectively. Polymorphic "A" allele frequency was significant ($p<0.005$) higher in diabetic group than control. The incidence of diabetes was significantly higher in both male and female subjects in diabetic as well as in control group with or without family history of diabetes. Highly significant associations of GA and AA variants were found with both semi urban and urban diabetic subjects than that of rural diabetic subjects. Although significant differences were not found in clinical parameters according to genotypic variants.

The genetic linkage analysis of selective genes (KCNJ11/TCF7L2/SLC22A1) suggested that polymorphism of EK/TT/GA, EK/TT/AA and KK/TT/GA alleles showed the higher risk in T2DM subjects. From the obtained results, it can be concluded that KCNJ11, TCF7L2 and SLC22A1 gene polymorphisms are strongly associated with T2DM in population of Bangladesh.

Chapter 1
Introduction



1. Introduction

1.1 Overview

Now a day, diabetes mellitus has reached epidemic proportions and affects more than 170 million individuals worldwide, where some 90% of diabetic individuals have type 2 (non-insulin-dependent) diabetes mellitus. Within this category, no more than 10% account for monogenic forms (Stumvoll et al., 2005). A diabetes classification based on individual types' etiology was proposed by the Experts Committee of the World Health Organization in 1999 and is now commonly accepted. With the progress of knowledge, particularly in genetics, the etiologic classification was gradually extended in the last decade (Maecki and Skupieñ, 2008). As the human genome sequence information is now available, it has been apprehended that the genetic studies will identify more candidate genes for type 2 diabetes. Investigations revealed that the genetic linked with type 2 diabetes is large and may contain hundreds, or even thousands, of genes. More than 60 potential candidate genes have been examined as type 2 diabetes susceptibility genes for their involvement in insulin action, insulin secretion, and adipose metabolism. Although variants have been identified in many of these, only a few have been recognized as associated with diabetes or impaired protein function. Thus, deficits in gene/protein function were caused by mutations, or polymorphisms may become clinically significant when coupled with other genetic or acquired defects. The correlation between the genotype and phenotype makes the task of finding diabetogenes a formidable one (Almind et al., 2001).

Type 2 diabetes is called a typical complex and polygenic disease because several common risk genetic alleles have been identified as linked to this disease. Three common variants named Lys23 of KCNJ11, Pro12 of PPARG, and the T allele at rs7903146 of TCF7L2 are predisposed to type 2 diabetes mellitus in several extensive studies. Each of these polymorphisms is only moderately predisposed to type 2 diabetes (Weedon et al., 2006). Thus far, most of the success in defining type 2 diabetes genes (diabetogenes) has been achieved by studying relatively rare forms of the disease (Almind et al., 2001).

Unique clinical features characterize monogenic forms of diabetes. In that case, applying a tailored treatment, assuring optimal correction of the genetic conditioned and metabolic

defects are possible. The selection of appropriate treatment methods, assessment of the prognosis, and diabetes complications mostly depend on the differential diagnosis of the diseases and play an essential role in diabetology (Maecki and Skupieñ, 2008). Numerous genetic subtypes of diabetes have been described since 1992, due to gene mutations and β-cell dysfunction. This knowledge mostly classified the previously clinically categorized maturity-onset diabetes of the young (MODY), permanent neonatal diabetes mellitus (PNDM), or transient neonatal diabetes mellitus (TNDM) by genetic subgroup. The identification of gene subgroups can ensure appropriate treatment, genetic counseling, and prognostic information. Although, previously MODY was clinically defined as an autosomal, dominantly inherited, non-insulin-dependent early-onset diabetes, now at least eight genetic subgroups including HNF4A, GCK, HNF1A, HNF1B, NEUROD1, KLF11, CEL, and PAX4 of MODY with discrete phenotype were established (Murphy et al., 2008; Nyunt et al., 2009).

Similarly, monogenic forms of neonatal diabetes (Murphy et al., 2008), occurred before six months of age, was categorized as either transient neonatal diabetes (TNDM), which accounts for 50 to 60 % of cases, or permanent (PNDM) neonatal diabetes (Rongrong et al., 2008; Aguilar-Bryan and Bryan, 2008). TNDM is a developmental disorder of insulin production that resolves postnatally. PNDM causes insulin secretory failure, which occurs in the late fetal or early postnatal period and does not go into remission (Michel and Hélène, 2007). More than 90% of TNDM cases are due to genetic origin. The majority (68 %) are attributable to abnormalities in the 6q24 region, whereas 10 and 13 % of cases are due to mutations in KCNJ11 and ABCC8, respectively. Whereas, ten genes involved in pancreatic development, β-cell apoptosis, or dysfunction have been recognized as responsible for the development of PNDM (Aguilar-Bryan and Bryan, 2008).

Therefore, the present study was carried out to investigate the distribution and association of KCNJ11, TCF7L2, and SLC22A1 gene polymorphisms in the development of T2DM and to evaluate the role of those gene polymorphisms with the glycemic response of drugs in diabetic patients of Bangladesh.

1.2 Diabetes Mellitus

Diabetes mellitus is commonly known as diabetes that causes several metabolic disorders of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin action, insulin secretion, or both (WHO, 2014a).

1.3 Glucose Metabolism and Diabetes Mellitus

After the digestion body breaks down the carbohydrates (e.g., sugars and starches) into glucose. Glucose is the form of sugar that can enter the blood stream. Cells throughout the body can absorb the glucose with the hormone insulin's help and use it for energy production. Diabetes develops when the body is unable to make enough insulin or cannot use insulin effectively or both. Insulin is made in the pancreas that contains clusters of cells called islets. Beta cells in the islets make insulin and release it into the blood. Insulin production in the beta cell of the pancreas and overview of type 2 diabetes mellitus is shown in **Figure 1.1**. If beta cells don't produce enough insulin or the body doesn't use the insulin that is present, glucose builds up in the blood. As a result, the blood glucose level increases that lead to prediabetes or diabetes.

Blood glucose levels higher than normal but not high enough to be diagnosed as diabetes are known as prediabetes conditions. High blood glucose or hyperglycemia damages nerves and blood vessels that may lead to complications such as heart disease, stroke, kidney disease, blindness, dental disease, and amputations (**Figure 1.2**). Other complications of diabetes may include increased susceptibility to other diseases (De Fronzo, 2009). The starting processes that cause diabetes are unknown, but scientists believe genes and environmental factors interact to induce diabetes in most cases (Grant et al., 2009).

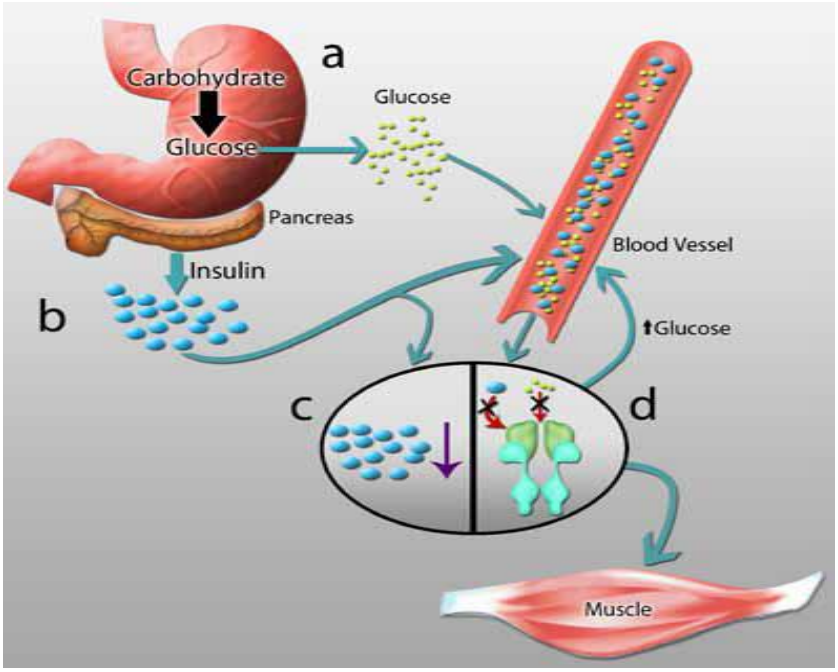


Figure 1.1: Overview of Type 2 Diabetes Mellitus

a) Carbohydrates, metabolized to glucose in the digestive tract, are released into blood stream, b) stimulating the pancreas to produces insulin, c). With insulin resistance, either the production of insulin decreases, or it cannot bind to its receptor, d) resulting in a decrease in glucose uptake by organs like muscles. This ultimately results in increased blood glucose levels and the development of T2DM

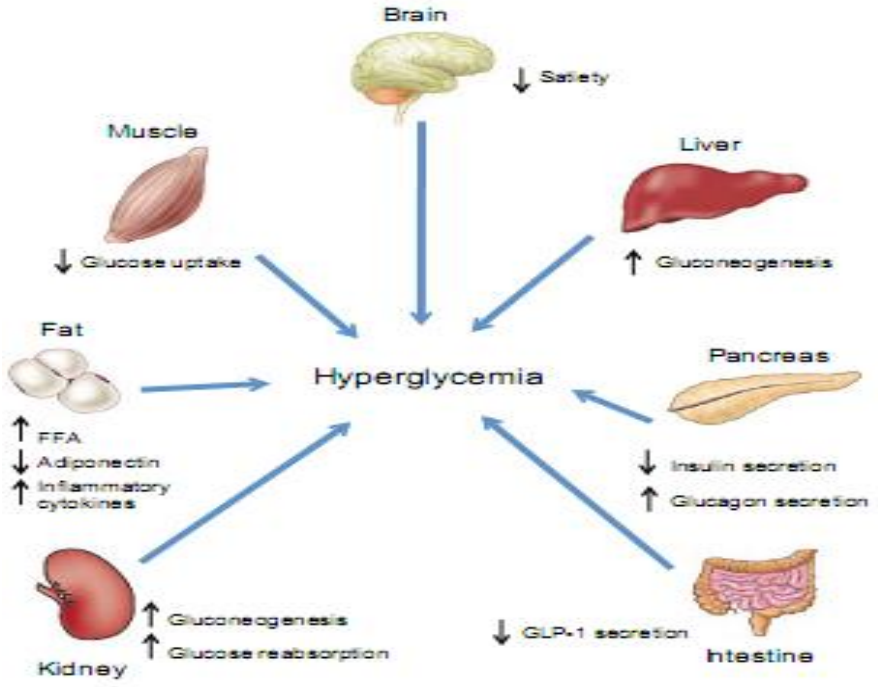


Figure 1.2: Multi-organ and Tissue Pathophysiology of Type 2 Diabetes

FFA; free fatty acids; GLP-1; glucagon-like peptide-1.

1.4 Lipid Profiles and Type 2 Diabetes

Unfavorable blood lipids have been reported as a risk factor for type 2 diabetes mellitus. There is an inverse relationship between HDL-cholesterol levels and risk of type 2 diabetes. Some studies found low HDL-cholesterol is a more potent risk factor for type 2 diabetes in women only. High plasma triglycerides and low plasma HDL cholesterol levels are both seen in the insulin resistance. So, non-fasting triglycerides and HDL cholesterol levels reflect the degree of insulin resistance (Tangvarasittichai et al., 2010; Tangvarasittichai, 2015).

Insulin resistance and type 2 diabetes mellitus are characterized by dyslipidemia, which is a condition characterized by a low HDL level and a high level of TG. Low HDL levels are associated with hyperinsulinemia or insulin resistance. Obesity, metabolic syndrome, and T2DM may also show the same dyslipidemia characteristics. On the other hand, TG, HDL, and TG/HDL ratios are independently associated with insulin levels, insulin resistance, and cardiovascular disease (CVD) risk. All of these features are related to coronary heart disease risk in obesity, metabolic syndrome, and T2DM patients (Tangvarasittichai et al., 2010; Tangvarasittichai, 2015; Li et al., 2008).

Therefore, fasting serum lipids profile should be determined in T2DM patients as recommended by the American Diabetes Association (ADA). ADA recommended for the satisfied lipids profile level as low-risk by LDL-C<100 mg/dL (2.6 mmol/L), triglycerides<150 mg/dL (1.7 mmol/L) and HDL-C>50 mg/dL (1.3 mmol/L) (Haffner, 2003).

Dyslipidaemia in individuals with type 2 diabetes is very common, with a prevalence of 72–85%. This phenomenon is associated with a significantly increased risk of coronary artery disease in diabetes patients. Lipid abnormalities observed in patients with type 2 diabetes play a central role in developing atherosclerosis and other cardiovascular diseases. These lipid abnormalities are not only quantitative but also qualitative and kinetic. The main quantitative lipid abnormalities of diabetic dyslipidemia are increased

triacylglycerols and reduced HDL-cholesterol (Turner et al., 1998), described in **Figure 1.3**.

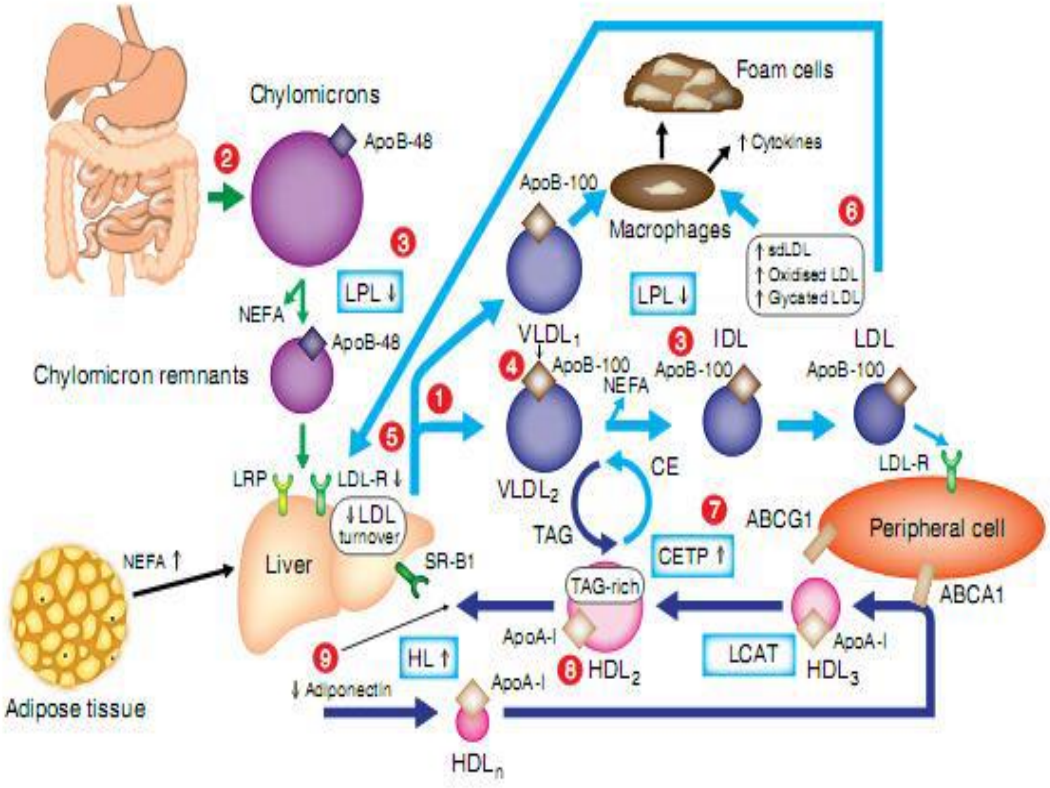


Figure 1.3: Main Lipid Abnormalities in Type 2 Diabetes

CE, cholesterol esters; CETP, cholesteryl ester transfer protein; dLDL, small, dense LDL; HDLn, nascent HDL; HL, hepatic lipase; HSL, hormone- sensitive lipase; LPL, lipoprotein lipase; sLDL-R, LDL receptor; SR-B1, scavenger receptor B1; TAG, triacylglycerol.

This mechanism is summarized as follows:

1. **Triacylglycerols** (hypertriglyceridemia, qualitative and kinetic abnormalities): (1) increased VLDL production (mostly VLDL1), (2) increased chylomicron production, (3) increased production of large VLDL (VLDL1), (4) reduced catabolism of both chylomicrons and VLDLs (diminished LPL activity), preferentially taken up by macrophages.

2. **LDL-cholesterol** (qualitative and kinetic abnormalities): (5) increased number of glycated LDLs, small, dense nLDLs (TAG-rich) and oxidized LDLs, (6) reduced

LDL turnover (decreased LDL B/E receptor), which are preferentially taken up by macrophages;

3. **HDL (low HDL-cholesterol**, qualitative and kinetic abnormalities): (7) increased CETP activity (increased transfer of triacylglycerols from TAG-rich lipoproteins to LDLs and HDLs), (8) increased TAG content of HDLs, promoting HL activity and HDL catabolism, (9) low plasma adiponectin favoring the increase in HDL catabolism (Verges, 2015).

1.5 The Global Burden of Type 2 Diabetes Mellitus

The International Diabetes Federation (IDF) estimates the number of adults with diabetes in the world about 381.8 million in 2013, a number that will expand to 591.9 million in 2035, including 202 million in the Asia-Pacific region, where the prevalence of diabetes is also predicted to be increased from 8.6% in 2013 to 11.8% in 2035. 138 million (36% of all patients) diabetic patients are in the Asia Pacific region. The increase of the prevalence of diabetes will be highest in sub-Saharan Africa, with a projected growth of 109.6%, from 19.8 million in 2013 to 41.5 million in 2035 (**Table 1.1)** (Seshasai et al., 2011; IDF Diabetes Atlas, 2013).

Table 1.1: IDF Regions and Global Projections of the Number of People with Diabetes 2013-2035

IDF REGION	2013 MILLIONS	2035 MILLIONS	INCREASE %
Africa	19.8	41.4	109%
Middle East and North Africa	34.6	67.9	96%
South-East Asia	72.1	123	71%
South and Central America	24.1	38.5	60%
Western Pacific	138.2	201.8	46%
North America and Caribbean	36.7	50.4	37%
Europe	56.3	68.9	22%
World	**381.8**	**591.9**	**55%**

The number of people with type 2 diabetes increases every country; 80% of people with diabetes live in low- and middle-income countries (**Figure 1.4)** (IDF Diabetes Atlas, 2013).

Figure 1.4: Prevalence (%) of Diabetes (20-79 years) by Income Group and Age

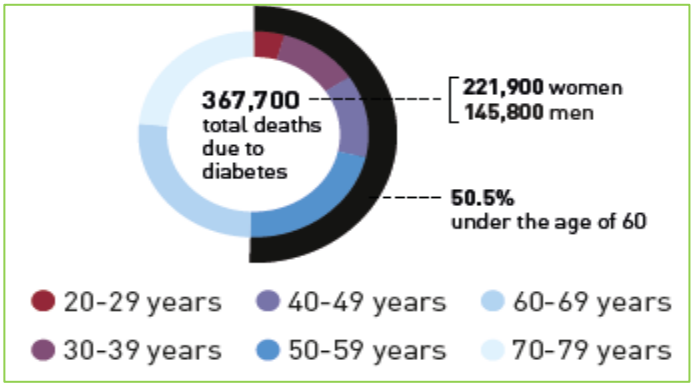


Figure 1.5: Deaths due to Diabetes by Age and Sex

The greatest number of diabetes people are between 40-59 years of age **Figure 1.5** (IDF 2013). 175 million people with diabetes are undiagnosed in the world. More than 79,000 children developed type 1 diabetes in 2013, and more than 21 million live births were affected by diabetes during pregnancy in 2013. Diabetes caused 5.1 million deaths in 2013; every six seconds, a person died from diabetes (IDF Diabetes Atlas, 2013). Because of its widespread prevalence and potentially devastating impact, DM has become an international and national priority area of health concern (WHO 2014b).

In the United States, the prevalence among adults was 5.3% in the late 1970s and 8.2% in the late 1990s, showing an increase of approximately 1.5-fold over the 20 years (Gregg et al., 2004). In Japan, the prevalence in 1970 was 1.6% in men and 0.9% in women, considerably lower than that in the U.S. during the same period. However, in 1990, the prevalence was 6.0% in men and 4.0% in women, showing that the prevalence had rapidly increased by approximately four times. In other East Asian countries, e.g., China, South Korea, and Malaysia, the prevalence increased 3 to 5 times from around 1980 to around 2000 **(Figure 1.6)** (Islam, 1999; Hu, 2011; Kim, 2011; Khalid et al., 1998).

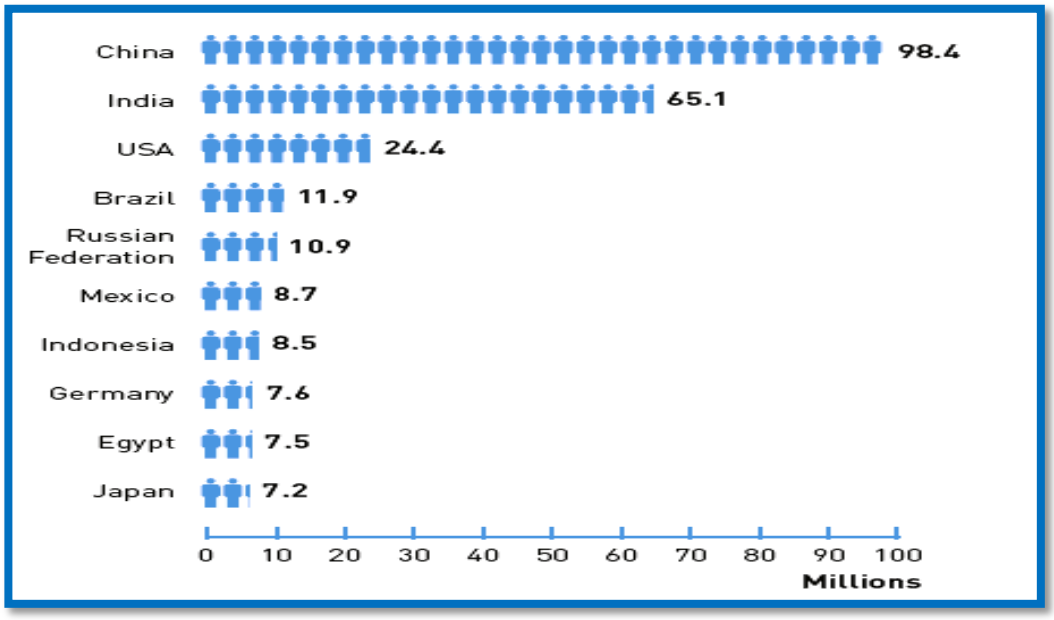


Figure 1.6 Top 10 Countries for Number of People with Diabetes (20-79 years), 2013

1.6 Prevalence of Type 2 Diabetes in Bangladesh

Diabetes morbidity and mortality are more prevalent in the Bangladeshi population. However, the exact causes or risk factors for diabetes are not known for increased mortality in Bangladesh. Both genetic predisposition and environmental factors are commonly attributed to irrespective of ethnicity. It puts a tremendous burden on individual health and the family, social, and country economy.

The number of registered diabetes in the year 1956 was 39, which has been increased to 15,296 in 1998 as found from diabetes registry in Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine, and Metabolic Disorders (BIRDEM). On average, 60 percent are male among the registered diabetic patients, 62 percent from urban, 32 percent from rural, and 6 percent from semi-urban. The overall estimated prevalence of diabetes in the Bangladeshi population is 5.6%, and in which more than 96 percent is reported to have type 2 diabetes (Shamima et al., 2014).

A report was published in 2011 by the International Diabetes Federation (IDF) which estimated that 8.4 million of diabetic people in Bangladesh with 8th position in the rank and it are expected to be 16.8 million by the year 2030 with 5th position in rank (IDF 2013) and 6.7 million of people living in Bangladesh is suffering from impaired glucose tolerance (IGT) unexpectedly. This explosion in diabetes prevalence will place in Bangladesh among the top 5 countries in terms of the number of people living with diabetes in 2030. Bangladesh is a United Nations-designated least developed country (LDC) with a disproportionately high diabetes population. Among all people living with diabetes in the 48 LDCs, more than one-third live in Bangladesh. 40% of all diabetes people in the least developed countries live in Bangladesh (Novo Nordisk, 2012). The distribution of people with diabetes in the least developed countries is shown in **(Figure 1.7).**

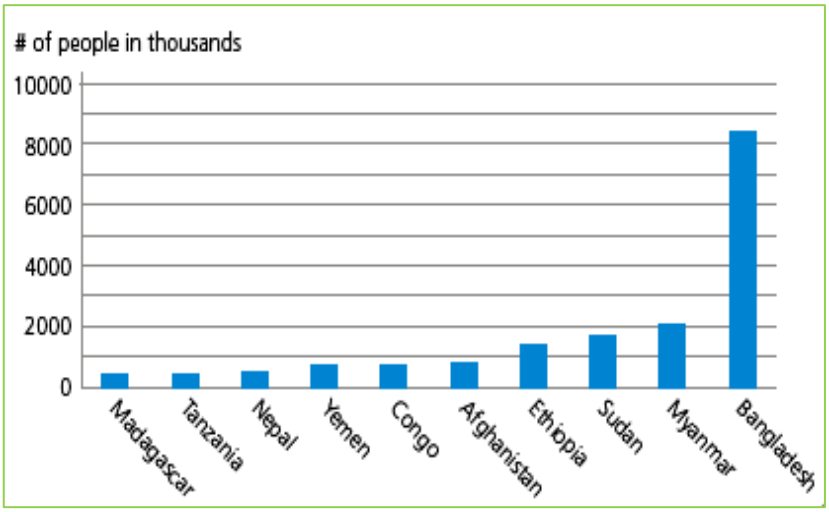


Figure 1.7: Distribution of People with Diabetes in the Least Developed Countries

In Bangladesh, which had a population of 149.8 million in 2011, a meta-analysis showed that the prevalence of diabetes among adults had increased substantially, from 4% in 1995-2000 to 5% in 2001-2005 to 9% in 2006-2010 respectively. According to the International Diabetes Federation, the prevalence will be 13% by 2030. In Bangladesh, diabetes is the tenth most expensive disease in total healthcare costs allocated for an illness (Khan et al., 1998; IDF, 2013; Bangladesh population and housing census, 2011). Studies suggested that type 2 diabetes prevalence is between 2% and 21% and the prevalence of metabolic syndrome between 3% and 20% (Zaman and Ahmed, 2006; Jesmin et al., 2012).

1.7 Types of Diabetes Mellitus

The old classification of diabetes divided it into juvenile- and maturity-onset diabetes. Now an etiology based classification devised by the American Diabetes Association (ADA) and the World Health Organization (WHO) is used that divides diabetes into type 1 diabetes (T1DM), type 2 diabetes (T2DM), monogenic diabetes, gestational diabetes, and other types of diabetes (ADA 2010). In type-1 diabetes, patients have little or no endogenous insulin, and therefore insulin therapy is required for survival. Type-2 diabetes mellitus is a disease in which insulin is abnormally secreted or does not act appropriately, which leads to elevated blood glucose, and prolonged high glucose levels can lead to multiple organ damage (Doria et al., 2008). A recent study investigates the association of diabetes with a broad range of health conditions. People with diabetes had a 25-75% higher risk of dying from cancer, infection, liver disease, lung disease, mental disorder, etc. Overall, approximately 40% of excess death in diabetic patients appears to be due to nonvascular events (Seshasai et al., 2011)

The genetic factor is one of the critical risk factors for diabetes. T2DM is a metabolic disorder that is an extremely heterogeneous multifactorial disease and involves a complex interaction of environmental factors and susceptibility genes (**Figure 1.8**). Monogenic diabetes is a term used for diabetes type caused by a mutation in a single gene.

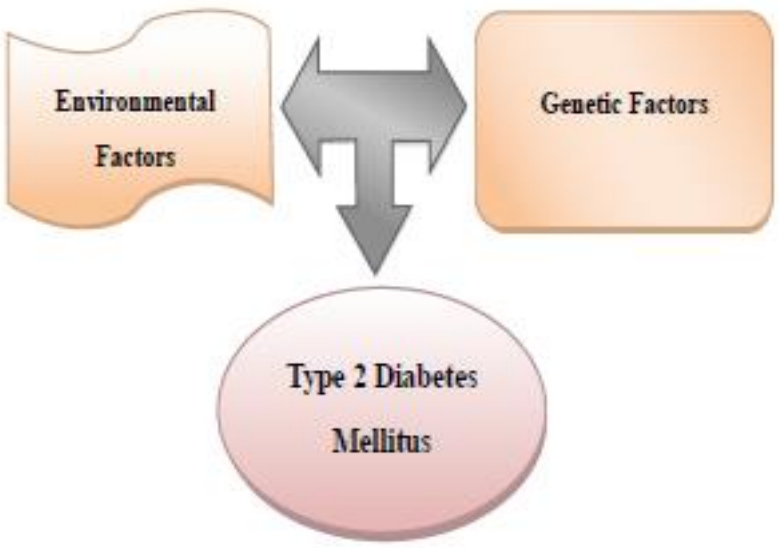


Figure 1.8 Factors Involved in the Development of T2DM

The mode of inheritance of T2DM is polygenic, i.e., polymorphism in many genes results in disease development. Type 2 diabetes may be maturity-onset diabetes of youth (MODY) 5-10% patients, latent adult-onset autoimmune diabetes (LADA) 5-10% patients, secondary to rare genetic disorders 5-10%, and remaining 70-85% patients are poorly defined. This 70-85 % is the typical T2DM (Type 2 diabetes mellitus) patients (Gerich, 1998).

1.8 T2DM is a Genetic Disease: Classical Evidence

Multiple lines of evidence support the view that genetic components play an important role in the pathogenesis of T2DM.

1.8.1 The Spectrum of T2DM Prevalence in Different Ethnic Groups

The disease prevalence varies substantially among ethnic groups that share a similar environment, supports the idea that genetic factors contribute to disease predisposition. It has been reported that the prevalence of T2DM was widely varied in a different ethnic population, such as 1% in Chile Mapuche Indian, 2% in Europe Caucasians, 41% in Nauru (Pacific Island), and 50% in Pima Indians of Arizona (Diamond, 2003). These ethnic differences may be due to non-genetic environmental and cultural factors.

1.8.2 Familial Aggregation

Familial aggregation of T2DM is another indication focused on the disease's genetic contribution, although families share similar environments, cultures, and habits. Nearly 4-fold increased risk for T2DM in siblings of a single affected diabetic parent and 6-fold for both parents affected compared to the general population indicated the role of genetic in familial aggregation (Meigs et al., 2000).

1.8.3 Twin Studies

Several studies with T2DM twin proposed concordance rates ranged from 0.29 to 1.00 in monozygotic (MZ) twins, while 0.10–0.43 in dizygotic (DZ) twins (Barnett et al., 1981; Newman et al., 1987; Poulsen et al., 1999; Medici et al., 1999). Concordance among both MZ and DZ twins increases with the follow-up period (Medici et al., 1999). Despite several restraints in twin studies, the high concordance in MZ twins and the 50% fall in DZ twins provide convincing evidence for a genetic ingredient of T2DM (Medici et al., 1999).

1.8.4 Heritability of Intermediate Phenotypes

It is well known that insulin sensitivity and insulin secretion worsen in parallel with T2DM conditions. Several studies with genetically identical co-twins of a diabetic proband concluded that both defects forecast the progression of T2DM and reported genetic basis for measures of both insulin sensitivity and insulin secretion (Elbein et al., 2000; Elbein et al., 1999; Gerich, 1998; Vaag et al., 1995).

1.9 Types of T2DM

Types of T2DM include maturity-onset diabetes of youth (MODY), latent adult-onset autoimmune diabetes (LADA), secondary to rare genetic disorders.

1.9.1 MODY

MODY is the old term based on the old classification of diabetes, was first described by Tattersall and Fajans (1995) for young diabetic patients treated without insulin for two years after diagnosis at age less than 25 years (Shields et al., 2010). It is the monogenic genetic form of T2DM, which is inherited in autosomal dominant mode (Kanwal et al.,

2011) and also a heterogeneous disorder characterized by impaired pancreatic beta-cells functions (Velho and Robert, 2002) **(Table 1.2)**.

Table 1.2: Sub-types of MODY

MODY Sub-Type	Gene Affected	Protein Affected	Locus	Gene Function	Primary Defect
Type 1	HNF4 alpha	Hepatocyte nuclear factor 4 alpha	20q	Transcription factor (Nuclear factor)	Pancreas
Type 2	GCK	Glucokinase	7p15-p13	Hexokinase IV	Pancreas/Liver
Type 3	TCF1	Hepatocyte nuclear factor 1 alpha	12q24.2	Transcription factor (Homeodomain)	Pancreas/Kidney
Type 4	IPF1	insulin promoter factor-1	13q12.1	Transcription factor (Homeodomain)	Pancreas
Type 5	TCF2	Hepatocyte nuclear factor 1 beta	17q12	Transcription factor (Homeodomain)	Kidney/Pancreas
Type 6	Neuro D1	Neurogenic differentiation factor 1	2q	Transcription factor (bHLH)	Pancreas
Type 7	KLF11	Kruppel-like factor 11	2p25	Transforming growth factor-beta inducible-early growth response 2	Pancreas
Type 8	CEL	Bile salt dependent lipase	9q34.3	Hydrolyze cholesterol esters as well as a variety of other dietary esters (OMIM 114840)	Pancreas
Type 9	PAX4	Paired domain gene 4	7q32	Transcription factor (paired domain gene 4)	Pancreas
Type 10	INS	Insulin	11p15.5	Beta cells of the islets of Langerhans	NF-kappa-B
Type 11	BLK	Tyrosine kinase B-Lymphocyte specific	8p23-p22	Tyrosine kinase (B lymphocytes)	MIN6 beta cells

MODY is classified into subtypes related to genes' involvement (Kanwal et al., 2011) **(Table 1.2)**. Till now, seven genes have been reported to be associated with MODY.

Frequencies of genetically associated genes with MODY differs in different populations, with 2 and 3 being predominant over others. Identification and knowledge about the dominant genes have improved our understanding of MODY's molecular and clinical diagnosis. Simultaneously, other genes with MODY X (Still unknown) need to be identified for recognizing the role MODY in T2DM (Ben et al., 2011).

1.9.2 LADA

Latent Adult-onset autoimmune diabetes (LADA) is a particular type of diabetes with a low progressive form, first characterized by Pittman et al. (1982) is often misdiagnosed as T2DM. Classification of LADA is difficult as it lies between type 1 and type 2 diabetes (Gale, 2005) and is usually classified as type 2 diabetes with GAD antibodies due to autoimmunity (Matejkova-Behanova, 2001). The presence of circulating glutamic acid decarboxylase-65 (GAD65) autoantibodies and/or islet cell antibodies is the LADA's critical diagnostic criteria for LADA (Matejkova-Behanova, 2001; Nambam et al., 2010). LADA is initially treated with diet control/oral hypoglycemics without using insulin injections several years after diagnosis (Fourlanos et al., 2005). As LADA shares the characteristics of both diabetes (type 1 and type 2) and involved genes of both type 1 (HLA, INS VNTR, and PTPN22) and type 2 (TCF7L2), it is considered as diabetes of admixture of T1DM and T2DM (Cervin et al., 2008).

1.9.3 Diabetes: Other Monogenic Forms and Secondary to Rare Genetic Disorders

Genes associated with other syndromes, monogenic forms, and rare genetic disorders have also been identified. The genes associated with these syndromes are shown in **Table 1.3**.

These genetic disorders include deafness, optic atrophy, Wolfram syndrome, renal and urogenital system structural anomalies, neurological, renal disease, DEND syndrome (developmental delay, epilepsy, and neonatal diabetes), partial lipodystrophy, congenital generalized lipodystrophy and skeletal lytic lesions etc. (Owen and McCarthy, 2007; Doria et al. 2008).

Table 1.3: Genes Associated with Monogenic Diabetes and Secondary to Rare Genetic Disorders

Gene Affected	Protein Affected	Locus	Gene Function	Primary Defect
WFS1	Wolframin	4p16.1	10 transmembrane domain protein, function unknown	Diabetes insipidus and mellitus with optic atrophy and deafness (DIDMOAD); Wolfram Syndrome
ZCD2	ERIS	4q22-q24	Zinc finger protein ZCD2	Wolfram Syndrome 2
INS	Insulin	11p15.5	Hormone	Mutation in insulin, proinsulin, and proinsulin processing
PTF1A	Pancreas transcription factor 1	10p12	Alpha subunit of PTF1	Permanent neonatal diabetes with cerebellar agenesis
EIF2AK3	PERK	2p12	Pancreatic eIF2-alpha kinase	Wolcott-Rallison Syndrome
Mitochondrial genome	MIDD	Mutation at 3243 mtDNA	tRNA for leucine	Maternally inherited diabetes and deafness; other mitochondrial mutation also observed
Mitochondrial genome		Mutation at 14709 mtDNA	tRNA for glutamic acid	Mitochondrial myopathy with diabetes
KCNJ11	Kir6.2	11p15.1	Potassium channel	Permanent and transient neonatal diabetes
ABCC8	Sur1	11p15.1	Sulfonylurea receptor	Permanent and transient neonatal diabetes
PLAGL1 (ZAC)/ HYMA1	Pleomorhpic adenoma gene 1; hydatidiform mole transcript	11p15.1	Plagl1 - Nuclear zinc finger protein	Imprinted region, exact gene unclear; transient neonatal diabetes type 1
INSR	Insulin receptor	19p13	Receptor tyrosine kinase	Insulin-resistant diabetes with various phenotypes: leprechaunism, Rabson-Mendenhall or type A syndrome
AKT2	PKB-beta	19q1	Serine-threonine kinase	Severe insulin resistance
LMNA	Lamin A/C	1q21	Inner nuclear membrane protein	Face-sparing partial lipodystrophy with peripheral fat loss; mutations also associated with cardiomyopathy; muscular dystrophy; and Hutchinson-Gilford Progeria
LMNB2	Lamin B2	19p13	Inner nuclear membrane protein	Partial lipodystrophy sparing legs (Barraquer-Simons Syndrome)
PPARG	Peroxisome proliferator activated receptor γ	3p25	Nuclear receptor for prostaglands and thiazolidine-diones	Rare variants in ligand binding Domain associated with insulin resistance, hypertension, buttock lipodystrophy
AGPAT2	1-acyl glycerol-3-phosphophate O-acyltransferase 2	9q34	Enzyme of phospholipid metabolism	Congenital generalized lipodystrophy with skeletal lytic lesions (Berardinelli-Seip Syndrome)
BSCL2	Seipin	11q13	398 amino acid protein of unknown function	Congenital generalized lipodystrophy, learning disabilities

1.10 Heterogeneous Multi-factorial T2DM

Because of the heterogeneity of T2DM and the involvement of multiple genes, 70-85% of poorly defined diabetic patients are typical T2DM patients (Yang et al., 2012). Although environmental factors play an essential role in the development T2DM, a genetic factor

also has a significant influence on the susceptibility of T2DM (Wheeler and Barroso, 2011).

Despite many efforts, the identification of susceptibility loci and genetic predisposition of T2DM had been a great challenge, until recent initiation and advancement of genome-wide association scans (GWAS), which provided a better understanding about T2DM susceptibility genes, identification of susceptibility loci, and pathogenesis of T2DM (McCarthy and Zeggini, 2009). With GWAS, which efficiently detects multiple small-effect common variants, identifying >70 new genetic loci showing significant associations with T2DM has been established and increased from 2 identified loci by older approaches. Many of these identified genes are involved in encoding proteins necessary for glucose metabolism, beta-cell function, and insulin secretion (Yang et al., 2012; Saxena et al., 2013). Susceptibility genes so far identified include HNF1B, WFS1, JAZF1, TSPAN8/LGR5, THADA, ADAMTS9, IGF2BP2, CDC123/CAMK1D, PPARG, KCNJ11, TCF7L2, FTO, HHEX/IDE, SLC30A8, CDKAL1, CDKN2A/2B, NOTCH2, and KCNQ1 (**Figure 1.9**). From these genes, only PPARG and KCNJ11 have been linked as a risk factor for T2DM (Prokopenko et al., 2008). However, there is still a limitation in GWAS to detect rare variants with a stronger effect. Mostly identified T2D loci are common variants with small effects (Sanghera and Blackett, 2012). Although, until now, the GWAS is the best method to detect and identify new additional genes and loci in various populations and providing a more detailed genetic understanding of the pathophysiology of T2DM.

By GWAS, the association of zinc transporter and member of solute carrier family SLC30A8, HHEX, TCF7L2, and KCNJ11 with T2D was first reported by Sladek et al. (2007), and within the same year, other researchers confirmed the association of these genes with T2D (Saxena et al., 2007; Scott et al., 2007; Welcome Trust Case Control Consortium 2007; Steinthorsdottir et al., 2007). A GWAS in the Japanese population was shown to be linked to 6 loci named KCNJ11, HHEX, SLC30A8, IGF2BP2, CDKN2A/B, and CDKAL1, which was previously reported, but no novel loci were identified. A meta-analysis of three T2D GWAS by the Diabetes Genetics Replication and Meta-analysis (DIAGRAM) consortium using 2.2 million SNPs identified additional six novel loci with a strong association, including CDC123, JAZF1, THADA, ADAMTS9, TSPAN8, and

NOTCH2 (Zeggini et al., 2008). Association of FTO gene reported for obesity (Frayling et al., 2007) previously was confirmed by this and other replication studies. Association of TCF7L2 gene was replicated with the identification of novel regions on chromosome 7, 18p, 2p, and 13p by GWAS in African Americans. The candidate genes in these regions are TCF7L1, VAMP5, VAMP8, CDK8, INSIG2, IPF1, PAX8, IL18R1, members of the IL1 and IL1 receptor families, and MAP4K4 (Elbein et al., 2009).

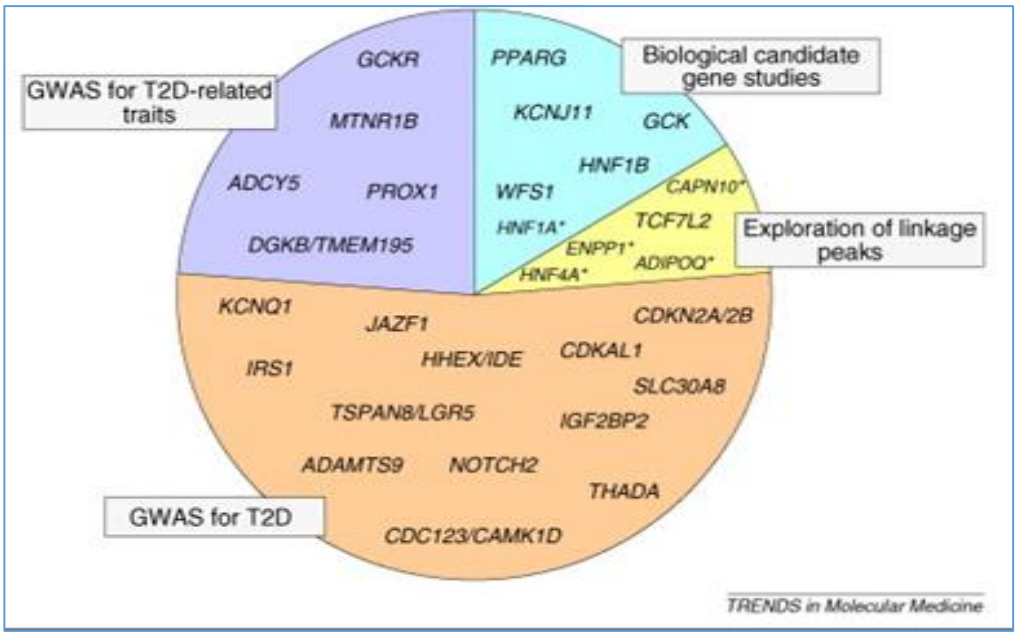


Figure 1.9: Some Genetic Variants Associated with Type 2 Diabetes Mellitus

GWAS showed that diabetes-associated variants in CDKAL1, CDKN2B, HHEX/IDE, IGF2BP2, KCNJ11, SLC30A8, and TCF7L2 account for 2.0-8.5% of the variance of T2DM-related traits (**Figure 1.9**) and have a role in disease etiology by physiological alterations leading to T2DM, such as glucose intolerance, impaired insulin secretion or insulin resistance. An extensive scale association analysis of 8,130 T2D cases and 38,987 controls of European descent identified some new T2D loci BCL11A, ZBED3, KLF14, TPS53INP1, CHCHD9, KCNQ1, CENTD2, HMGA2, HNF1A, ZFAND6, PRC1 extending to chromosome X gene DUSP9. These identified loci affect insulin action and β cell function and show evidence of association of genes involved in the cell cycle to T2D (Voight et al., 2010).

The majority of GWAS for T2D has been performed on the European population. The GWAS that has been carried out in the non-European population, especially those with unique cultural and demographic histories and biological traits, like population originating from the Indian subcontinent (Pakistan, India, and Bangladesh) so far are limited to provide a better insight into the 'genetic landscape of disease' in these populations. GWAS results in other populations cannot be used to predict risk in the South Asian community. A small number of studies carried out so far replicated some previously reported loci and identified some new susceptibility locus that was not reported to be associated with T2D in other populations (Rees et al., 2011).

A meta-analysis of the European ancestry population, including Caucasian, identifies new locus KCNQ1 (rs 231362) and HNF1A (overlap between monogenic and multifactorial forms of diabetes). The new KCNQ1 locus was confirmed by a later study in India and the USA (Voight et al., 2010; Been et al., 2011). Replication of GWAS validated variants in the Pakistani population of Mirpur, Azad Kashmir region resulted in the replication of 13 variants, including KCNQ1, JAZF1, IRS1, KLF14, CHCHD9, and DUSP9 that were not previously reported to be associated with T2D in the South Asian population (Rees et al., 2011).

A meta-analysis of 39 multiethnic population identifies SREBF1, TH/INS (study-wide significance $p<2.4X10^{-6}$), and GATAD2A/CILP2/PBX4 (genome-wide significance $p=5.7X10^{-9}$) as additional locus with one that was already known loci suggesting considerable overlap across various ethnic groups (Saxena et al., 2012). Meta-analysis was carried out, including 34,840 cases and 1,14,981 controls of European Descent and Pakistan, to understand genetic architecture and pathogenesis of T2D better. The study identified ten new, unreported T2D loci and two that showed sex-differentiated association (ZMIZ1, ANK1, KLHDC5, TLE1, ANKRD55, CILP2, MC4R, BCAR1, HMG20A, and GRB14 respectively) (Morris et al., 2012). A recent GWAS study in Punjabi Sikhs from India identified a novel locus in the SGCG gene (rs9552911) contributing to T2D susceptibility along with associations at HMG1L1/CTCFL, PLXNA4, SCAP, and chr5p11 (Saxena et al., 2013).

A meta-analysis of two Hispanic studies reported the association of T2DM with two known genes, HNF1A and KCNQ1, and the unreported C14orf70 (Parra et al., 2011). Two novel loci (PTPRD and SRR) associated with T2DM susceptibility were identified in the Han Chinese population in a two-stage GWAS. The study also showed the involvement of KCNQ1 that showed susceptibility to T2DM in Japanese, European, and Hispanic people (Tsai et al., 2010). Linkage to chromosome 6q21-q23 and 1q21-q24 was reported in Chinese and Northern European Caucasians in two separate studies (Xiang et al., 2004; Das et al., 2004). In African American families, susceptibility was found in genes VAMP5, VAMP8, TCF7L1, IPF1, PAX8, IL18R1, MAP4K4, CDK8, and members of IL1 and IL1R families by genome-wide linkage (Elbein et al., 2009). IGF2BP, CDKN2A, KCNJ11, HHEX, SLC30A8, and CDKAL1are associated with T2DM in the Japanese population (Hu et al., 2009). These genes are strong candidates associated with T2DM susceptibility in various ethnicities, as reported in different GWAS (Omori et al., 2008). Hepatocyte nuclear factor-4 (HNF4) is a transcription factor that plays a vital role in the expression of glucose metabolism genes that have been associated with T2DM in the Ashkenazi Jewish population and Finland families (Love-Gregory et al., 2004; Silander et al., 2004). Polymorphisms of the TCF7L2 gene associated with T2DM susceptibility has been reported in diverse ethnicities like European, West African, Danish, Icelandic, East Asian, French Dutch, Caucasian, and Brazilian, etc. (Sladek et al., 2007; Helgason et al., 2007; van Vliet-Ostaptchouk et al., 2007; van Hoek et al., 2008; Marquezine et al., 2008). Variations in other type 2 associated genes like TFAP2B (Maeda et al., 2005), Calpain-10 (first gene to be identified associated with T2DM susceptibility in 1996) (Horikawa et al., 2000), KCNJ11 (Yang et al., 2012), FTO, HHEX, IGF2BP2, SLC30A8, and PPARG discovered in GWAS are also associated with gestational diabetes in women of Korean ancestry (Cho et al., 2009). The role of inflammation in the pathogenesis of type 2 diabetes was studied by Hu et al. (2004) in women, and their study showed that Elevated C-reactive proteins were a strong predictor of T2DM and arbitrate TNF-R2 and IL-6 association with T2DM. A meta-analysis of the European population showed a novel association of X-chromosomal locus near DUSP9 and HNF1A that is implicated in both monogenic and polygenic T2DM. These loci control and regulate insulin action and β-cell function. This

study also proved the involvement of genes of the cell cycle, indicating the influence of unrelated traits in the development of T2DM (Ruchat et al., 2009).

Table 1.4: Genetic Variants Associated with Type 2 Diabetes Mellitus (Parchwani et al., 2013)

Name of Genes	Functions	References
TCF7L2, Transcription factor 7-like 2	Encodes a high mobility group box- containing transcription factor that plays a key role in Wnt signaling pathway.	Song et al., 2004
PPARG, Peroxisome proliferator-activated receptor γ	Transcription factor involved in adipocyte development.	Elbein, 2007
KCNJ11, Potassium inwardly-rectifying channel, subfamily J, member 11	Potassium channel that is part of the sulfonylurea receptor complex.	Tsuchiya et al., 2006
WFS1, Wolfram syndrome 1	Endoplasmic reticulum transmembrane protein expressed in the brain, heart and β-cells.	Haffner, 1998
HNF1B, Hepatocyte nuclear factor-1β	Transcription factor involved in pancreatic development.	Elbein et al., 1999
SLC30A8, Solute carrier family 30 (zinc transporter), member 8	Expressed in β-cells – it is a Zinc transporter, this being necessary for insulin storage in secretory granules and being part of the secretory mechanism.	Horikawa et al., 2000
HHEX, Hematopoietically expressed homeobox	Encodes a member of the homeobox family of transcription factor involved in pancreatic development.	Song et al., 2004
CDKAL1, CDK5 regulatory subunit associated protein 1-like 1	The protein product shares homology with CDK5 regulatory subunit–associated-protein-1, a neuronal protein that inhibits activation of CDK-5.	Poulsen et al., 1999
IGF2BP2, IGF-2 mRNA binding protein 2	Regulates IGF-2 translation by binding to the 5′ UTR of IGF-2 mRNA; pancreatic development.	Elbein, 2007
CDKN2A/B, Cyclin-dependent kinase inhibitor 2A and 2B	Function as cell growth regulators that control cell cycle G1 progression by inhibiting CDK; islet development.	Weedon et al., 2003
FTO, Fat mass and obesity associated	Affects fat mass, thereby indirectly predisposing to type 2DM	Luo et al., 2001

JAZF1, Juxtaposed with another zinc finger gene,	Encodes a nuclear protein with three zinc fingers; functions as a transcriptional repressor.	Groop et al., 1996
CDC123-CAMK1D, Cell division cycle 123 homolog (S. cerevisiae) and Calcium /calmodulin-dependent protein kinase 1D	CDC123 is a putative regulator of the cell cycle, while CAMK1D is a protein kinase that may be important in response to chemokines.	Song et al., 2004
TSPAN8-LGR5, Tetraspanin 8, and Leucine-rich repeat-containing G protein-coupled receptor 5	Tetraspanin 8 is a cell surface glycoprotein that complexes with integrins regulating development and growth. Lgr5 is a potential marker of intestinal stem cells and hair follicles in humans. It is a target of Wnt signaling.	Turner et al., 2005
THADA, Thyroid adenoma associated	Thyroid adenoma; associates with PPARG	Wild et al., 2004
ADAMTS9, ADAM metallopeptidase with thrombospondin type 1motif, 9	A member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) protein family expressed in muscle and pancreas.	Elbein et al., 2007
NOTCH2, Notch homolog 2	Transmembrane receptor implicated in pancreatic organogenesis.	Zeggini et al., 2008
KCNQ1, Potassium voltage-gated channel, KQT-like subfamily, member 1	Pore-forming subunit of voltage-gated K-channel (KvLQT1); risk allele impairs insulin secretion.	Ferrannini, 1998
IRS1, Insulin receptor substrate	Plays a crucial role in transmitting signals from the insulin & IGF-1 to the intracellular pathway.	Wild et al., 2004
MTNR1B, Melatonin receptor 1B	Encodes one of two high-affinity forms of a receptor for melatonin; risk allele associated with insulin secretion.	Tsuchiya et al., 2006
PROX1, Prospero protein homeobox 1	Corepressor of hepatocyte nuclear factor 4α, which plays a vital role in β-cell development	Ferrannini, 1998
GCKR, Glucokinase regulator	Regulatory protein that inhibits glucokinase	Wild et al., 2004
ADCY5, Adenylate cyclase 5	Formation of adenylate cyclase.	Elbein, 2007
UBE2E2, Ubiquitin-conjugating enzyme E2F2	-	Elbein, 2007
BCL11A, B-cell lymphoma/	Encodes C2H2 type zinc-finger protein.	Wild et al., 2004

leukemia 11A		
GCK, Glucokinase	Three tissue-specific forms phosphorylate glucose to produce glucose-6-phosphate in the liver and the β-cell.	Groop et al., 1996
DGKB/TMEM195, Diacylglycerol kinase beta and Transmembrane protein 195	DGKB encodes an isotype of DAG kinase, which increases DAG and therefore increases insulin secretion. TMEM195 is a membrane phosphoprotein.	Groop et al., 1996
C2CD4A/BC2, calcium-dependent domain containing 4B	Encodes nuclear-localized factor 2, which is expressed in endothelial cells and the endocrine and exocrine pancreas.	Ghosh et al., 2000
KLF14, Kroppel like factor 14, also known as Basic transcription element binding protein (BTED5)	Regulates the transcription of various genes, including TGF βR11.	Groop et al., 1996

1.11 Pharmacogenomics of T2DM

Pharmacogenomics is the field to study the relationship between drug response effects due to an individual's genetic variation that will help create personal medicine adapted to one's genetic content. There are so many studies that have now elaborated on the effect of polymorphisms on different drug responses. Response to T2DM treatment varies depending on complications, disease duration, and the treatment based on pharmacogenomics will reduce the risk before the symptoms of T2DM appear. Nowadays, most diabetic medications are not based on specific molecular targets and disease pathogenesis knowledge. Understanding pathogenic mechanisms have led to discovering new avenues of drug targets **(Figure 1.10)** (Moller, 2011; Vella, 2009).

Nine classes of drugs have been approved to treat diabetes with exercise regimens and diet control, including insulins, sulfonylureas, glinides, biguanides, and α-glucosidase inhibitors thiazolidinediones, glucagon-like peptide one mimetics, amylin mimetics, and dipeptidyl peptidase four inhibitors (Reitman and Schadt, 2007). However, still, there is no single medication that can maintain an optimal glycemic level. Polymorphisms in TCF7L2, PPARG, cytochrome P450 drug-metabolizing enzymes, etc. modify drug response to metformin, sulphonylureas, DPP4 inhibitors, thiazolidinediones, and meglitinides (Holstein et al., 2011).

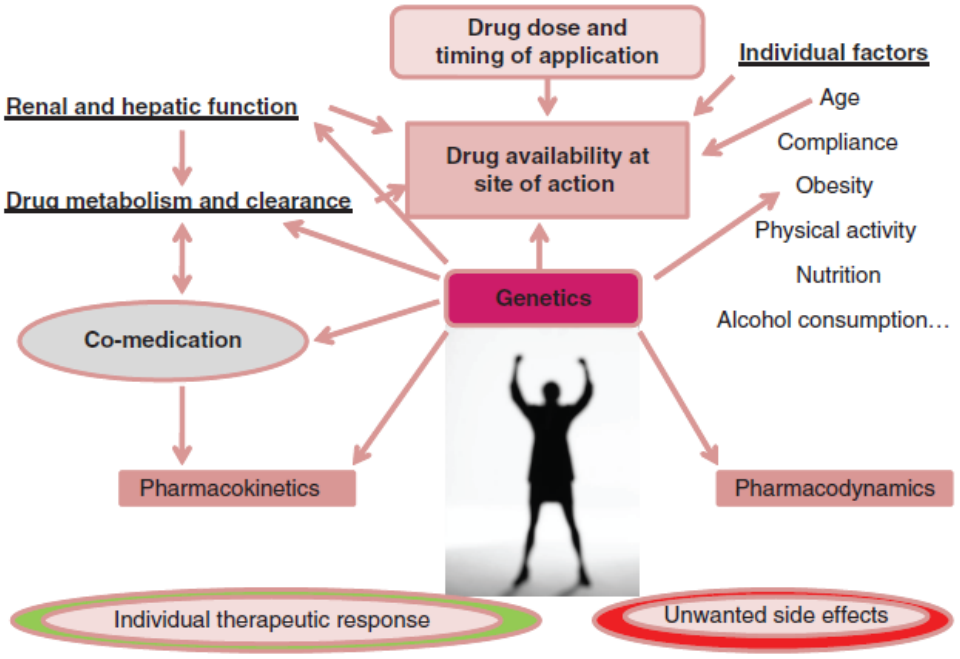


Figure 1.10 Complex Interactions of Various Factors Resulting in Individual Therapeutic Drug Response and Side Effects

PPARG and KCNJ11 the first variants that reproduced the site of action for thiazolidinediones and sulfonylureas, respectively. Variation in TCF7L2 has been found to be associated with a change in insulin secretory response to GLP-1 and variation in HbA1C level after the introduction of sulfonylureas. On the other hand, Shu et al. (2007) reported that polymorphism in organic cation transporter-1 (OCT-1) decreased the response to metformin while another study conducted by Becker et al. (2009) was in contrast to that study. Many other studies have also been reported, but still, there is a relative lack of studies in pharmacogenetics of T2DM and many other diseases (**Table 1.5)** (Shu et al., 2007; Becker et al., 2009; Avery et al., 2009; Aquilante, 2010; Distefano and Watanabe, 2010; Lebovitz, 2011)**.**

Table 1.5: Polymorphisms Affecting Drug Response

Drug	Mechanism of Action	Transporter gene/protein	Response affecting Polymorphism
Sulfonylureas	Increase β-cell insulin secretion by a glucose-independent mechanism	Not studied	CYP2C9 *2/*2, *2/*3, *3/*3, TCF7L2, E23K variant of KCNJ11
Meglitinides	Increase β-cell insulin secretion by a glucose-independent mechanism	SLCO1B1	CYPC8*1/*3, CYP2C9*3
DPP4 inhibitors	Increase insulin secretion by a glucose-dependent mechanism Suppress glucagon secretion by a glucose-dependent mechanism	Incretin hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)	TCF7L2, MTNR1B
GLP1 receptor agonists	Increase insulin secretion by a glucose-dependent mechanism Suppress glucagon secretion by a glucose-dependent mechanism	GLP receptor	T149M polymorphism
Thiazolidinediones	Decrease insulin resistance	SLCO1B1 (OATP1B1)	CYP2C8*3 and *4, PPARG Ala12
Metformin	Decrease insulin resistance	SLC22A1 (OCT1) SLC22A2 (OCT2)	SNP rs622342 in SLC22A1 gene encoding OCT1
α-Glucosidase inhibitors	Act within the gastrointestinal tract to lower postprandial glucose excursions		Pro12Ala of PPARG, Gly482 of PGC1A, TT genotype of APM1 polymorphism +276 G/T

(Partly adapted from Schroner et al. [68] and Holstein et al. [61]

However, complex factors and genetic heterogeneity make it challenging to study genetic factors in pharmacotherapy and personalized medicines. Further advances in GWAS and pharmacogenetics will reveal new genetic variants that modify drug response to diabetes and the development of first-line therapy (Schroner et al., 2011; Wolford et al., 2004).

1.12 Background of Investigated Diabetes Associated Genes

1.12.1 KCNJ11 Gene

The KCNJ11 gene, a member of the potassium channel gene family, is located at 11p15.1 and has no intron (**Figure 1.11**). The physical map is shown in **Figure 1.12**. This gene encodes an inward-rectifier potassium ion channel (Kir6.2). The Kir6.2 protein binds with the high-affinity sulfonylurea receptor 1 (SUR1) and forms the KATP channel. SUR1 is encoded by the ABCC8 gene located next to the KCNJ11 gene. The Kir6.2 protein is a 390-amino acid protein with two transmembrane domains (M1 and M2) and intracellular N- and C-terminals. Structurally, Kir6.2 tetramers form the pore, and four high-affinity SUR1 subunits surround the pore of the KATP channel located at the plasma membrane of pancreatic beta cells. This channel modulates insulin production and secretion through glucose metabolism (Taggart et al., 2010).

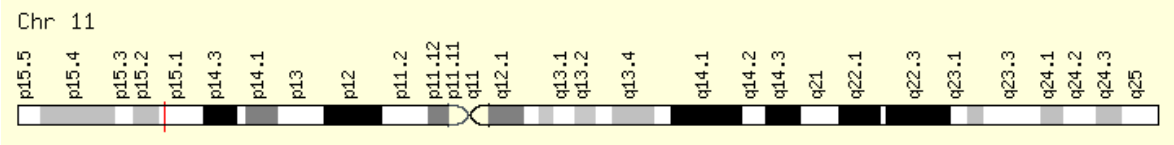


Figure 1.11: Chromosomal Location of KCNJ11 Gene

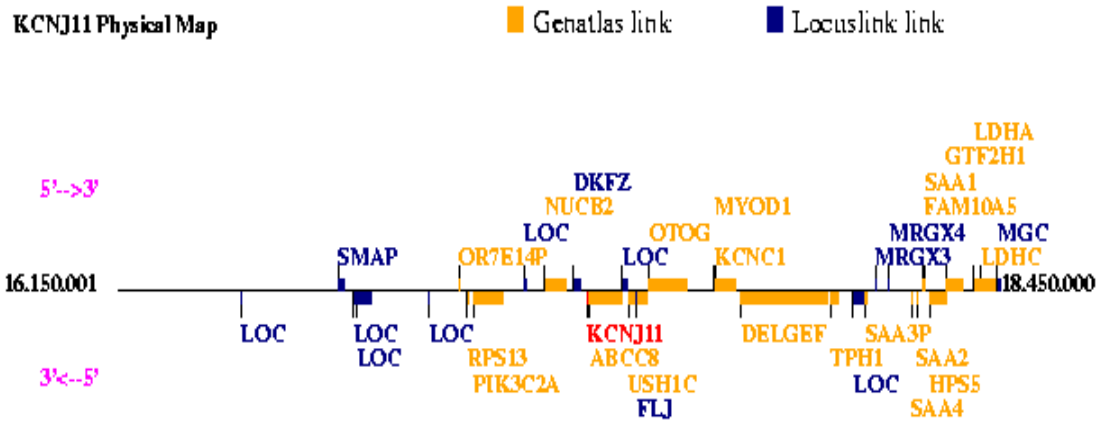


Figure 1.12: Physical Map of KCNJ11 Gene

The KCNJ11 and ABCC8 genes are next to each other on chromosome 11p15.1. KCNJ11 is a single exon (dark box) gene encoding the Kir6.2 protein, and ABCC8 has 35 exons (dark boxes) encoding the SUR1 protein; both are subunits of the ATP-sensitive potassium (KATP) channel. Metabolism of glucose can affect ATP levels and, thereby, the functions of the KATP channel (**Figure 1.13**).

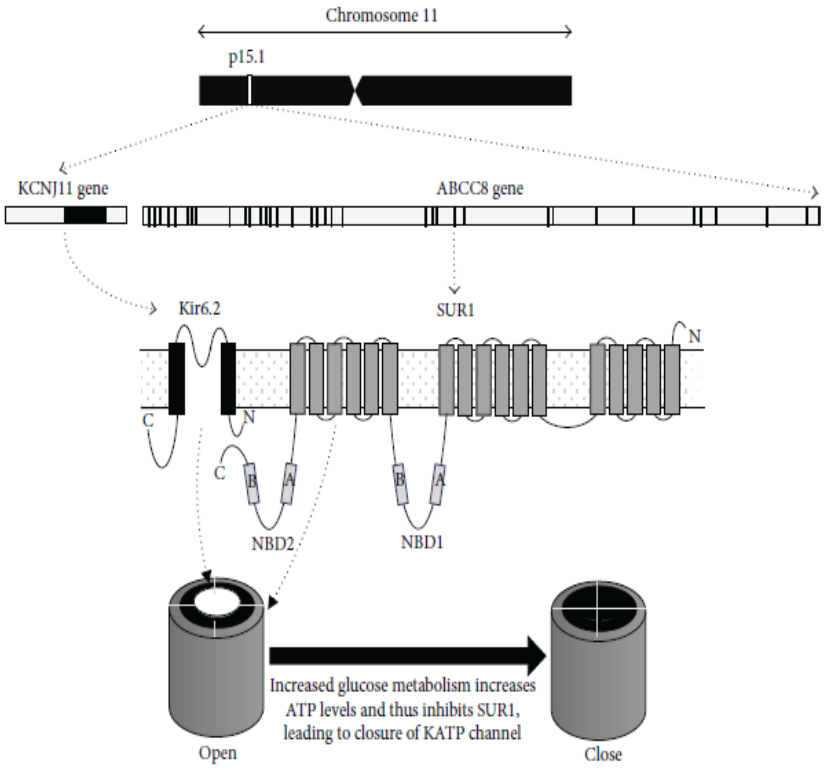


Figure 1.13: ABCC8 and KCNJ11 Genes and their Encoded Proteins and Functions

ABCC8: ATP-binding cassette transporter subfamily C member 8; KCNJ11: potassium inwardly-rectifying channel, subfamily J, member 11; Kir6.2: inward-rectifier potassium ion channel; SUR1: sulfonylurea receptor 1; NBD1: nucleotide-binding domain 1; NBD2: nucleotide-binding domain 2; N: NH2 terminal of protein; C: COOH terminal of protein; A: Walker A motif; B: Walker B motif; cAMP: cyclic adenosine monophosphate; ATP: adenosine triphosphate.

1.12.1.1 Role of Kir6.2 in Insulin Secretion

The Kir6.2 proteins, coupled with the SUR1 protein in the KATP channel, mediate insulin secretion and involve a wide range of physiological responses. Increased glucose induces higher potassium flow into the cell through the KATP channel. ADP in the presence of magnesium (Mg) converts to ATP to close the KATP channel by binding to Kir6.2, increasing the intracellular potassium ion concentration, which depolarizes the cell

membrane and subsequently activates the calcium ion (Ca2+) channel. Ca2+ is a ubiquitous intracellular second messenger critical for cellular functioning and influences the voltage-dependent potassium channels to repolarize the cell membrane, leading to the voltage-dependent calcium channels' closure. Increased intracellular free Ca^{2+} levels trigger other components of the insulin secretion pathway to release granules at or near the plasma membrane (**Figure 1.14**). Mutations in the KCNJ11 gene can cause DM because of ATP's reduced ability to inhibit the KATP channel activity and the enhanced ability of MgATP to stimulate this channel's function simultaneously. This is associated with defective insulin secretion, ultimately causing DM (Ashcroft, 2006).

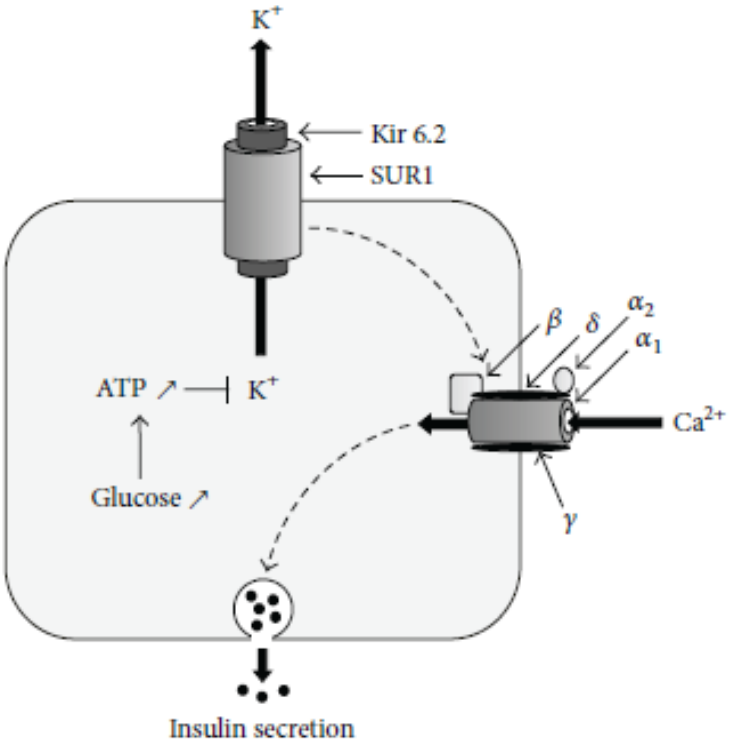


Figure 1.14 Mechanism of Insulin Secretion by the KATP Channel in Pancreatic β-Cells

The Kir6.2 and SUR1 proteins in the KATP channel mediate insulin secretion. An increase in glucose levels stimulates the KATP channel to allow the entry of potassium ions. An increase in intracellular potassium ions depolarizes the cell membrane and induces calcium channels to increase intracellular free Ca2+ levels. The calcium ions trigger other components of the insulin secretion pathway to release granules at or near the plasma membrane KATP: ATP-sensitive potassium channel; Kir6.2: inward-rectifier potassium ion channel; SUR1: sulfonylurea receptor 1; ATP: adenosine triphosphate; K+: potassium ion; Ca2+: calcium ion. The calcium channel is composed of α1, α2, β, γ, and δsubunits.

1.12.1.2 Role of KCNJ11 Genetic in the Development of Diabetes

People with a family history of T1DM and T2DM are six and three times more likely, respectively, to develop these diseases than are unrelated individuals (Dorman and Bunker, 2000). Multiple genes are involved in DM. Those that have garnered the most attention are the ATP-binding cassette transporter subfamily C member 8 (ABCC8) gene, the KCNJ11 gene, and the peroxisome proliferator-activated receptor-gamma (PPARG) gene. Most of these genes are involved in insulin action/glucose metabolism, pancreatic beta-cell function, or other metabolic conditions (e.g., energy intake/expenditure, lipid metabolism) (Schwenk et al., 2013). Mutations in genes such as ABCC8 and KCNJ11 can disrupt the potentiation activity of the KATP channel and have thus been associated with permanent neonatal DM (Abujbara et al., 2014). The PPARG gene is implicated in adipogenesis and the development of insulin resistance. Deleterious mutations in this gene impair insulin resistance and cause a lack of insulin (Pattanayak et al., 2014). More than 60, 500, and 65 loci have been identified from recent genome-wide association studies for susceptibility to T1DM, T2DM, and GDM, respectively (Hivert et al., 2014). SNPs are the most common genetic variation within or outside a gene region in the human genome. SNPs' frequency is less than 1% in the genome, and approximately 54% of these variants are not deleterious (Mitchell et al., 2005). SNPs can modify the risk of disease, either alone or in the linkage, in one gene or in neighborhood genes. For instance, the common Pro12Ala polymorphism in the PPARG gene, the Glu23Lys polymorphism in the KCNJ11 gene, or the Ser1369Ala polymorphism in the ABCC8 gene was confirmed to be associated with DM (Bailey-Wilson and Wilson, 2015).

1.12.1.3 KCNJ11 Common Polymorphisms and Susceptibility with Diabetes

KCNJ11 has 219 SNPs, six of which have been receiving more attention for their association with diabetes. Among these six common SNPs, three are located in the coding regions, and three in the noncoding areas (**Table 1.6**). These six SNPs include rs5219, rs5215, rs5210, rs5218, rs886288, and rs2285676.

Table 1.6: Characteristics of KCNJ11 Gene Variants in Association with Diabetes

Number	SNP	Location		MAF	Allele	Amino acid	Diabetes	Association
		Chromosome	Gene					
1	rs2285676	17386478	3′ UTR	0.46	T>C	—	T2DM	Yes
2	rs5210	17386704	3′ UTR	0.46	G>A	—	T2DM	Yes No
3	rs5215	17387083	Exon	0.28	G>A	Val250Ile	T2DM T1DM GDM	Yes No No No
4	rs5218	17387522	Exon	0.27	C>T	Ala103Ala	T2DM	No
5	rs5219	17388025	Exon	0.27	G>A	Lys23Gln	T1DM T2DM GDM	No Yes No Yes No
6	rs886288	17389616	5′ near gene	0.46	T>C	—	T2DM	Yes

MAF: minor allele frequency; T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus; GDM: gestational diabetes mellitus; SNP: single nucleotide polymorphism; UTR: untranslated region.

1.12.1.3.1 KCNJ11 rs5219.

This locus is located in exon 1 of the KCNJ11 gene. Substitution of A to C (AAG→CAG) changes the amino acid from lysine to glutamine (Lys23Gln) at the NH_2-terminal tail of Kir6.2. Lysine has a positively charge epsilon-amino group, whereas glutamine is uncharged under all biological conditions. It does not make a remarkable change in the structure and function of the KCNJ11 protein despite this amino acid substitution theoretically (Harakalova et al., 2012). However, several studies have shown that the rs5219 variant may alter the ATP-binding region's charge and decrease channel sensitivity to ATP. Many studies showed a strong association between the rs5219 polymorphism and susceptibility to T2DM (Abdelhamid et al., 2014; Chen et al., 2013, Zhou et al. 2009), whereas some studies did not confirm positive finding (Keshavarz et al., 2014, Danquah et al., 2013; Gamboa et al., 2012). The meta-analysis showed that the rs5219 polymorphism is a risk factor for developing T2DM in Caucasians and in some Asian populations. East Asia populations were more prone to this disease, where the A allele frequency in most

patients was more common than in controls. Therefore, the genetic background can affect susceptibility to T2DM (Qiu et al., 2014).

The rs5219 polymorphism can affect the insulin secretion pathway where "A" allele of this locus impairs this pathway by reducing ATP sensitivity of the KATP channel, resulting in overactivity of the channel subsequent suppression of insulin secretion. This effect on insulin secretion is more significant in carriers of the AA genotype than carriers of the GA genotype (Liu et al., 2006). Similar results were observed for fasting plasma glucose and postprandial plasma glucose levels in patients with T2DM. The A allele increased the fasting plasma glucose and postprandial plasma glucose levels in these patients, whereas GA carriers had higher 2hrs postprandial plasma glucose levels than GG carriers with T2DM (Gonen et al., 2012; Shaat et al., 2005). This allele was also associated with a reduction in serum insulin levels in a post-oral glucose tolerance test (Yu et al., 2010).

1.12.1.3.2 KCNJ11 rs5215.

The rs5215 polymorphism is a nonsynonymous variant and is located in exon 1 of the KCNJ11 gene. A substitution of G causes it to A (GTC→ATC), which changes the amino acid from valine to isoleucine at residue 250. Valine is hydrophobic, whereas isoleucine is one of three amino acids having branched hydrocarbon side chains. Isoleucine is usually interchangeable with leucine and occasionally with valine in proteins. Of 13 studies on DM, 3 showed strong associations between this variant and T2DM (Chavali et al., 2011; Liu et al., 2006; Sakamoto et al., 2007), whereas the remaining studies showed no association with T2DM, T1DM, or GDM (Koo et al., 2007; Hotta et al., 2014; Kurzawski et al., 2012). In another study, Koo et al. (2007) reported that the rs5215 polymorphism was associated with blood pressure among subjects with T2DM.

1.12.1.3.3 KCNJ11 rs5210.

The rs5210 polymorphism is located at a highly conserved 3′ untranslated region (UTR) of the KCNJ11 gene. Reports relevant to susceptibility to T2DM, two identified a plausible role in the development of this disease, whereas the other studies did not confirm this relationship (Koo et al., 2007. Liu et al., 2006). A study found that this variant improves gliclazide's clinical efficacy in patients with T2DM (Murray and McLachlan, 2009). This

locus is a target of miR-1910; however, the mechanism of action of this miRNA in the development of DM is unknown. MiRNAs encompass 17 to 25 nucleotides, which post-transcriptionally regulate the expression of thousands of genes in a broad range of organisms in both normal physiological and disease contexts. Appropriate secretion of insulin from pancreatic beta cells is a vital factor in blood glucose homeostasis, and miRNAs have been identified as being involved in the regulation of insulin exocytosis, control insulin synthesis and release it in beta cells. The G allele is a potential target for miR-1910, whereas the A allele abolishes this miRNA's binding to this region (Dehwah et al., 2012; Alvarez and DiStefano, 2013).

1.12.1.3.4 KCNJ11 rs5218.

The rs5218 polymorphism is located in the 3′-UTR of the KCNJ11 gene. It is a synonymous variant with a substitution of G to A (GCC→GCT), which encodes for alanine at residue 103, a hydrophobic and ambivalent amino acid. There is only one report of this locus in DM, which showed no association with T2DM risk (Koo et al., 2007).

1.12.1.3.5 KCNJ11 rs886288 and rs2285676.

The rs886288 polymorphism is located in the 5′flank near the gene, whereas the rs2285676 polymorphism is located in the 3′-UTR region. Two studies revealed an association of the rs886288 andrs2285676 polymorphisms with T2DM (Koo et al., 2007. Liu et al., 2006).

1.12.2 TCF7L2 Gene

Transcription factor 7-like 2 (T-cell specific, HMG-box), also known as TCF7L2 or TCF4, is a protein acting as a transcription factor. In humans, this protein is encoded by the TCF7L2 gene (Castrop et al., 1992). The single nucleotide polymorphism (SNP) within the TCF7L2 gene, rs7903146, is, to date, the most significant genetic marker (Vaquero et al., 2012) associated with Type 2 diabetes mellitus (T2DM) risk. SNPs in this gene are linked to a higher risk of developing type 2 diabetes (Jin and Liu, 2008) and gestational diabetes (Zhang et al., 2013).

1.12.2.1 Function

TCF7L2 is a transcription factor influencing the transcription of several genes, thereby exerting many functions within the cell. It is a member of the Wnt signaling pathway. Stimulation of the pathway leads to the association of β-catenin with BCL9, translocation to the nucleus, and association with TCF7L2 (Lee et al., 2006), which in turn results in the activation of Wnt target genes, specifically repressing proglucagon synthesis in enteroendocrine cells (Jin and Liu, 2008).

1.12.2.2 Nomenclature

While TCF4 is sometimes misled as an alias symbol for TCF7L2, it is also officially approved by the HUGO Gene Nomenclature Committee for the transcription factor 4 gene. Transcription factor binding sites by QIAGEN in the TCF7L2 gene promoter:

- STAT3
- PPAR-gamma2
- PPAR-gamma1
- HNF-4alpha1
- N-Myc
- AP-2alpha

1.12.2.3 Genomic Location for TCF7L2 Gene

- Chromosome:10
- Start: 112,950,219 bp from pter
- End: 113,167,678 bp from pter
- Size: 217,460 bases
- Orientation: Plus strand

1.12.2.4 Genomic View for TCF7L2 Gene

Genes around TCF7L2 on UCSC Golden Path with GeneCards custom track Cytogenetic band:

- 10q25.2 by Ensembl
- 10q25.3 by Entrez Gene
- 10q25.3 by HGNC

The genomic location and physical map of the TCF7L2 gene are shown in **Figure 1.15 and 1.16.**

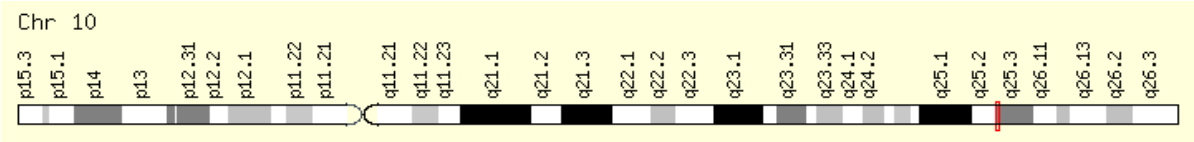


Figure 1.15: Chromosomal Location of TCF7L2 Gene

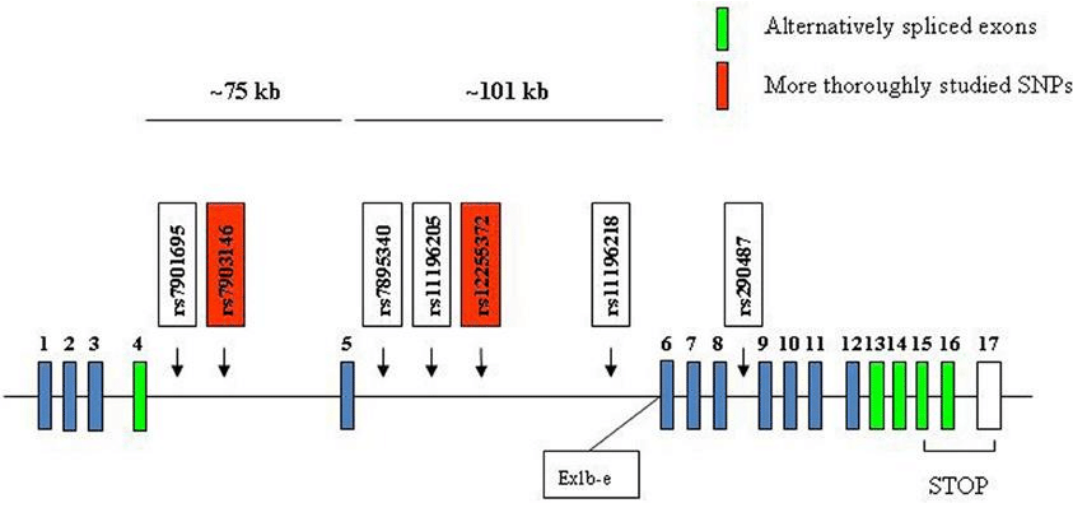


Figure 1.16: Physical Map of TCF7L2 Gene

1.12.2.5 TCF7L2 and its Role in β-Cell Function

TCF7L2, the susceptibility gene with the most considerable effect on disease susceptibility discovered to date, was identified pregenome-wide association with rapid replication of its consequence on diabetes susceptibility in multiple populations (Grant et al., 2006; Anna et al., 2009; Groves et al., 2006; Cauchi et al., 2006; Damcott et al., 2006; Weedon, 2007). TCF7L2 was a positional candidate gene that mapped to a region of genetic linkage to type 2 diabetes in the Icelandic population on chromosome 10. However, the identified TCF7L2 risk allele, which was present in ~28% of control subjects and ~36% of type 2 diabetic individuals, could not explain this linkage, so the finding was serendipitous (Grant et al., 2006). The specific genetic defect that causes the association of TCF7L2 with type 2 diabetes remains unclear. There are many highly correlated variants, none of which are

apparent functional candidates that show association with diabetes (Grant et al., 2006). The most likely candidate is the single nucleotide polymorphism rs7903146, which shows the strongest association with diabetes and resides in a noncoding region with no apparent mutational mechanism. However, it is clear that the effect of the TCF7L2 risk allele is through a defect in insulin secretion (Weedon, 2007).

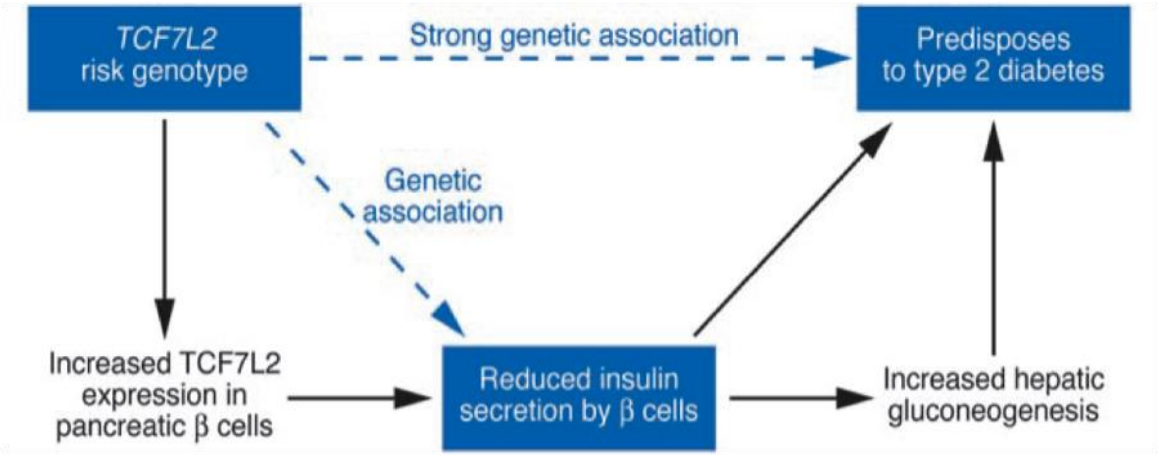


Figure 1.17 Regulation of Insulin Secretion from Pancreatic β-cells by TCF7L2 Gene

There have been few studies investigating the role of TCF7L2 on insulin secretion in isolated islets. Shu et al. (2008) reported that the silencing of TCF7L2 by siRNA resulted in strong suppression of insulin secretion in human and mouse islets. Conversely, overexpression of TCF7L2 stimulated insulin secretion. Exactly how TCF7L2 protein levels modulate insulin secretion was not established in the study by Shu et al. (2008).

The normal regulation of insulin secretion from pancreatic β-cells is well understood (**Figure 1.17)** (Rorsman and Renström, 2003). As reported by da Silva XG et al. (2009), the effects of over-expression and knockdown of TCF7L2 on β-cell function using an impressive battery of cell physiological techniques. They confirm that the silencing of TCF7L2 exerts a strong inhibitory effect on glucose-induced insulin secretion. By contrast, insulin secretion triggered by high extracellular K^+ was not affected. Unlike what was seen in the study of Shu et al. (2008), overexpression of TCF7L2 did not affect insulin secretion. The inhibition of glucose-stimulated insulin secretion produced by TCF7L2 silencing was not associated with any lowering of $[Ca^{2+}]_i$; if anything, the glucose

responses were larger in cells lacking TCF7L2. This suggests that glucose sensing in the β-cell was unaffected.

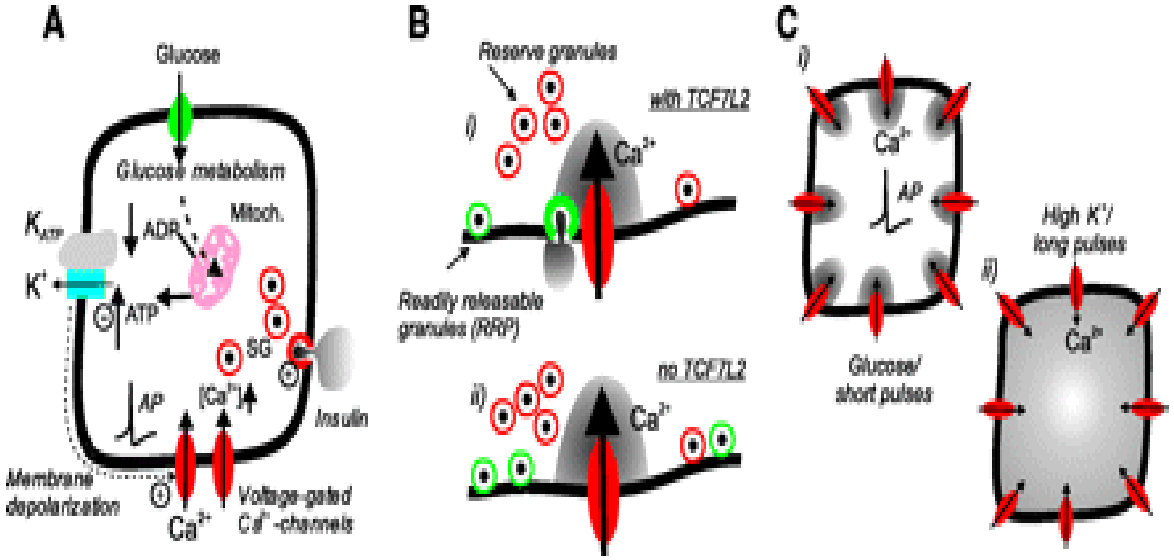


Figure 1.18: Mechanisms by which TCF7L2 Silencing Reduces Glucose-stimulated Insulin Secretion

A: *Stimulus-secretion coupling of the β-cell. Glucose, via mitochondrial (mitoch.) production of ATP and an increased ATP-to-ADP ratio, causes closure of ATP-sensitive K^+ (K_{ATP}) channels and elicits action potentials (APs) that are associated with the opening of voltage-gated Ca^{2+} channels. The increase in $[Ca^{2+}]_i$ stimulates exocytosis of insulin-containing secretory granules (SGs).*

B: *The insulin granules belong to different functional pools, which differ with regard to release competence. The vast majority of granules did not attain release competence and belong to a reserve pool (red granules). A small fraction of the granules is immediately available for release: the readily releasable pool (RRP) (green granules). Many readily releasable pool granules are situated in close proximity of the voltage-gated Ca^{2+} channels (i). In the absence of TCF7L2, the Ca^{2+} channels may detach from the secretory granules, and $[Ca^{2+}]_i$ increases in the wrong part of the β-cell (ii).*

C: *Localized increases in $[Ca^{2+}]_i$ (gray zones) close to the Ca^{2+} channels during brief action potential–like stimulation (i) and the global elevation produced during protracted (e.g., high K^+) stimulation (ii).*

Given these functional data, the inhibitory action of TCF7L2 gene silencing on glucose-induced insulin secretion seems paradoxical in so far as no part of the β-cell stimulus-

secretion coupling was perturbed in a way that would suppress insulin release. This might indicate that the distal events involved in the secretory granules' fusion might be involved (although the finding that insulin secretion elicited by high K^+ was unaffected seemingly militates against this notion). This would be consistent with the observation that TCF7L2 silencing affected the levels of the exocytotic proteins syntaxin-1 and munc18-1. It is unlikely. However, that reduced expression of munc18-1 alone is responsible for the observed defect because knockdown of this protein in β-cells inhibited both glucose- and high-K^+–induced secretion and interfered with granule docking (**Figure 1.18A)** (Tomas et al., 2008). The authors further investigated the secretory defect by capacitance measurements of the increase in cell surface area resulting from the granule membranes' addition to the plasma membrane. When the β-cells were depolarized for 2.5 s, the inhibitory effect was limited to 40%. However, responses to depolarizations as short as 0.2 s were reduced by 80%. This is significant because the β-cell action potential is <100 ms (Ribalet and Beigelman, 1980). The observation that the inhibitory effect of TCF7L2 silencing was particularly strong for the brief depolarization suggests that TCF7L2 somehow affects the distribution of voltage-gated Ca^{2+} channels **(Figure 1.18B)**. There is evidence that interference with the association of Ca^{2+} channels and secretory granules principally affects exocytosis triggered by brief stimuli in β-cells (Barg et al., 2001). This would be in agreement with the observations that high extracellular K^+ evokes insulin secretion. The latter condition produces a global elevation of $[Ca^{2+}]_i$. It triggers exocytosis of all release-competent granules at the plasma membrane regardless of whether they are associated with the voltage-gated Ca^{2+} channels **(Figure 1.18C).**

1.12.2.6 TCF7L2 and its Role of Insulin Secretion

With one of the first significant detailed studies, da Silva XG et al. (2009) reported the mechanisms by which the reduced expression of diabetes gene TCF7L2 affects insulin secretion and how the reduction of expression inhibits insulin secretion remains to be determined. As the diabetes susceptibility variants of TCF7L2 is situated in the noncoding region and most strongly associated with diabetes, Jin and Liu (2008) reported that they cause disease susceptibility by influencing the expression levels. Further, the finding that the silencing of TCF7L2 was strongly associated with insulin secretion inhibition

suggested that diabetes results from reduced TCF7L2 expression. Therefore, the report that the expression of TCF7L2 in type 2 diabetes is associated with a fivefold increase in TCF7L2 mRNA levels than non-diabetic human islets was unexpected (Lyssenko et al., 2007). The changes in TCF7L2 expression were likely to considerably exceed those observed in carriers of the at-risk T-allele of TCF7L2. Future studies will be required to assess how more subtle variations in TCF7L2 level impact on β-cell function. Besides, studies investigating the temporal and specific TCF7L2 isoform expression profiles in human islets are essential to clarify this key transcription factor's role in islet development and function.

1.12.3 SLC22A1 Gene

Human organic cation transporter 1 (OCT1) belongs to the largest superfamily of transporters with 12 transmembrane domains and the solute carrier family encoded by the SLC22A1 gene (Jonker and Schinkel, 2004). This transporter involves translocating a large variety of organic cations, including drugs ((**Figure 1.19**) (Koepsell, 2004).

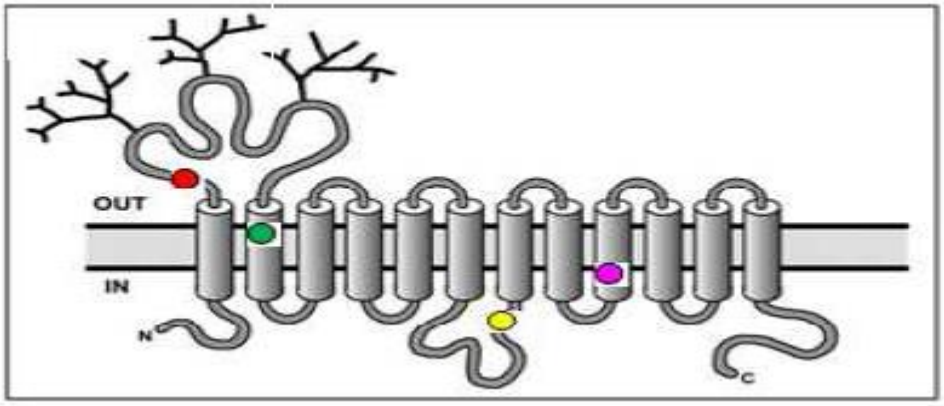


Figure 1.19: Predicted Secondary Structure of OCT1

The protein is thought to Contain 12 Transmembrane Domains with both N and C Terminus Located Intra Cellularly. The First Large Extracellular Loop Contains Three Putative N-Linked Glycosylation Sites (Indicated by Branches) Cytoplasmic (IN) and Extracellular (OUT) Orientation are: **Indicated ● Ser52Ser; ● Leu160Phe; ● Pro341Leu; ● Met408Val**

In humans, OCT1 is expressed in the basolateral membrane of hepatocytes and is the primary mediator of hepatic substrates (e.g., drugs) uptake (Gorboulev et al., 1997, Wang

et al., 2002). Indeed, in SLC22A1 (-/-) mice, significantly less anti-diabetic drug (e.g., Metformin) is distributed to the liver compared to control mice (Wang et al., 2002). SLC22A1, located at chromosome 6q25.3 (**Figure 1.20**), is adjacent to SLC22A2 (encoding OCT2) and SLC22A3 (encoding OCT3). It is consists of 11 exons spanning approximately 37 kb (Verhaagh et al., 1999). It was showed that human SLC22A1 is highly polymorphic in ethnically diverse populations (Sakata et al., 2004; Shu et al., 2007).

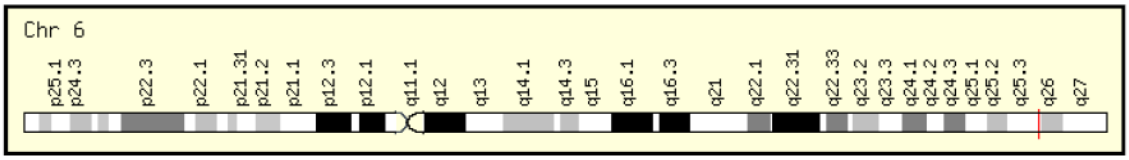


Figure 1.20: Chromosomal Location of SLC22A1 Gene

Many non-synonymous polymorphisms of SLC22A1, mostly found in Caucasian populations, exhibit reduced cellular assay activity. From Caucasians, Kerb et al. (2002) showed 25 polymorphisms, including eight, were non-synonymous, three of which showed decreasing activities. However, genetic variants of SLC22A1 that are related to reduced metformin uptake (i.e., S14F, R61C, S189L, G220V, G401S, 420del, G465R) have been identified primarily in populations with European ancestries and have not been identified in Asian American, Chinese, Korean, and Japanese populations (Song et al., 2008).

1.12.3.1 Function

Grundemann et al. 1994 performed functional assays, which suggested that OCT1 is the leading organic cation uptake system in hepatocytes and has standard features with organic cation uptake over the basolateral membrane-proximal renal tubules. Zhang et al. (1997) observed that Xenopus oocytes expressing OCT1 showed increased cation uptake.

1.12.3.2 Type 2 Diabetes Susceptibility Associated SLC22A1 Gene

A strong association of the SLC22A1 gene with T2DM were found in Javanese-Indonesian (Vitarani et al., 2019; Ningrum et al., 2019), Latvian (Tarasova et al., 2012); Slovenian (Klen et al., 2014), and Chinese Han population (Zhou et al., 2015) respectively. In one study with 33 Japanese patients with type 2 diabetes mellitus, two SLC22A1 polymorphisms (intron1 –43T>G, M408V), which do not exhibit altered function, were shown to have no significant effects on the clinical efficacy of metformin (Shikata et al., 2007). Kerb et al. (2002) reported significantly reduced (P341L) transport activities using tetraethyl-ammonium as a substrate.

1.13 Purposes for Selection of Genes

Many common genetic variants of TCF7L2, KCNJ11, and SLC22A1 have been shown to be associated with risk for T2DM. Individually, each of these polymorphisms is only moderately predisposed to type 2 diabetes. Thus, these variants have been studied in association with the development of T2DM in various ethnic groups. However, there are no studies about the role of TCF7L2, KCNJ11, and SLC22A1 genetic variants in the development of T2DM in the Bangladeshi population. Therefore, the present study was carried out with an aim to evaluate the association of TCF7L2, KCNJ11, and SLC22A1 gene polymorphisms with lipid concentrations, insulinemic status, and T2DM in a Bangladeshi population. Further, the study was done to understand the involvement of genetic polymorphisms in the development of T2DM.

1.14 Hypothesis

In the past decade, genome-wide association studies (GWAS) have dramatically increased the number of common single-nucleotide polymorphisms (SNPs) with confirmed association with T2DM. From a genome-wide panel, we selected some single nucleotide polymorphisms (SNPs) hypothesized to be more likely to modulate the risk of T2DM. It was hypothesized that- "SNPs in TCF7L2, KCNJ11, and SLC22A1 genetic variants could be associated with the risk of T2DM".

1.15 Aims and Objectives of the Study

Polymorphisms of TCF7L2, SLC22A1, and KCNJ11 genes has been observed in type 2 diabetic patients. Genetic variations of TCF7L2, SLC22A1, and KCNJ11 genes are considered to be involved in developing diabetes and oral hypoglycemic drug response. The presence of specific gene variants also induces total cholesterol, LDL, HDL, and TG in serum. Therefore, these gene variants have been studied in association with type 2 diabetes in different ethnic groups. However, there are no studies about the role of TCF7L2, SLC22A1, and KCNJ11 genetic variant in developing type 2 diabetes in the Bangladeshi population. Therefore, the present study was carried out to evaluate TCF7L2, SLC22A1 and KCNJ11 polymorphism with glycemic, lipidemic, insulinemic abnormalities and type 2 diabetes in Bangladeshi population.

1.15.1 Specific Objectives of the Study

The specific objectives of the study are as follows:

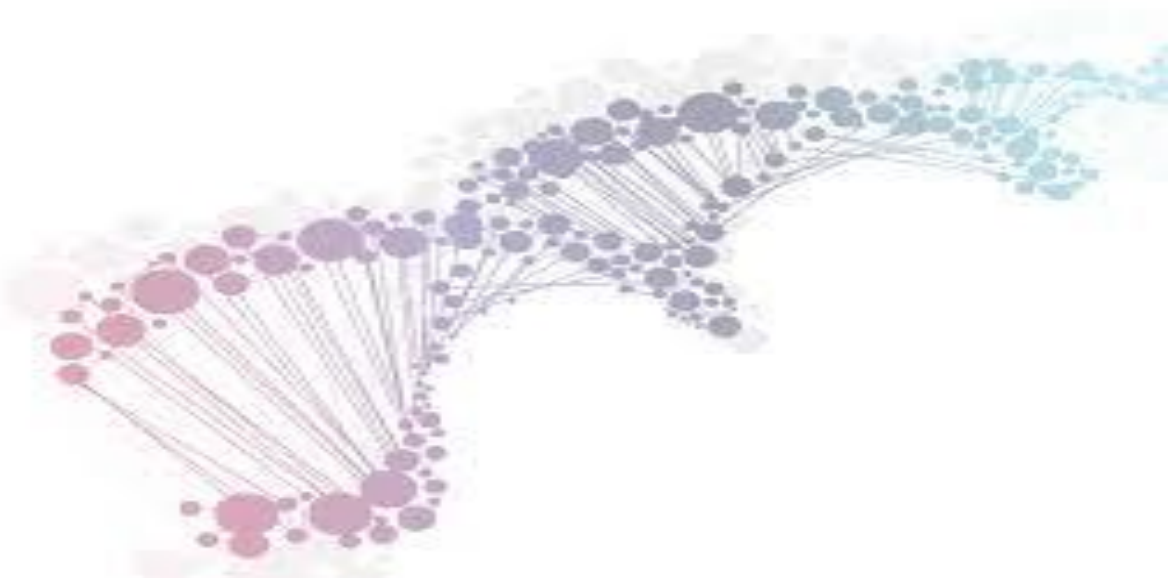
1. To investigate the association of polymorphisms of TCF7L2, SLC22A1, and KCNJ11 genes to the susceptibility of type 2 diabetes mellitus.
2. To identify different polymorphic variants of TCF7L2, SLC22A1, and KCNJ11 genes related to type 2 diabetes mellitus.
3. To identify the differences between genotype frequencies of the TCF7L2, SLC22A1, and KCNJ11 variants between type 2 diabetic and non-diabetic subjects.
4. To access the combined effect of these polymorphisms to modulate the risk of type 2 diabetes in Bangladesh.
5. To evaluated the association of lipid profile with the TCF7L2, SLC22A1 and KCNJ11 variants.
6. To evaluated the association of insulin, the Homeostasis Model of Assessment-insulin Sensitivity (HOMAS%), Beta-cell function (HOMAB%), Insulin Resistance (HOMA-IR), and Quantitative Insulin Sensitivity Check Index (QUICKI) with TCF7L2, SLC22A1 and KCNJ11 variants.

1.16 Significance of the Study

This study will evaluate the association of TCF7L2, SLC22A1, and KCNJ11 gene polymorphisms with type 2 diabetes in the Bangladeshi population. To the best of our knowledge, there were no studies so far done to investigate the role of TCF7L2, SLC22A1, and KCNJ11 genetic variant in the development of type 2 diabetes in the Bangladeshi population. It has been hypothesized that the Bangladeshi population is genetically more prone to develop diabetes and its complications. The increased prevalence of diabetes poses a severe problem in the healthcare system of Bangladesh. So, if the genetic polymorphisms that are responsible for increased susceptibility to type 2 diabetes could be determined, protective and preventive measures could be taken.

Chapter 2

Materials and Methods



2. Materials and Methods

2.1 Study Design: The study was designed as a case-control study.

(i) Case: Type 2 diabetes subject

(ii) Control: Healthy subject without any history of type 2 diabetes or other chronic diseases.

The study was designed to identify the variation in the genetic polymorphisms, which may be a risk factor for the development of diabetes.

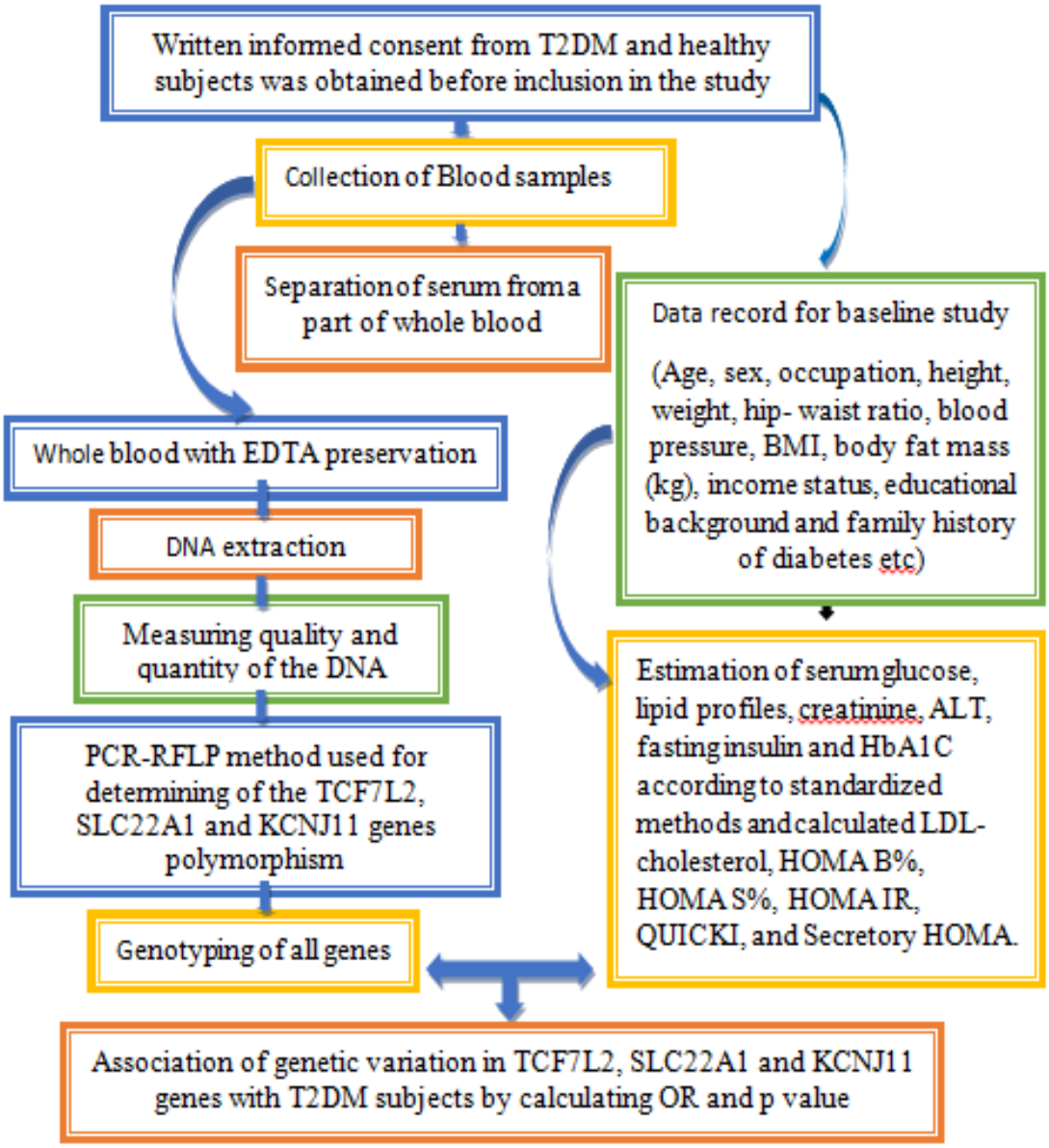


Figure 2.1: Experimental Design of the Study

2.2 Ethical Issue and Consent

After approval by the institutional Ethics Review Committee (ERC) of Bangladesh Institute of Research & Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) and Department of Biochemistry and Molecular Biology, University of Dhaka **(Appendix I and II),** and the study was conducted according to the declaration of Helsinki and Finland, (2013). The nature of the study was explained to all participants. They were also informed that their identity would not be disclosed, any time they can withdraw themselves from the study, and the generated data would be used only for research purposes. After signing a "Consent Form" the participants were included in the study, and blood was collected **(Appendix III)**.

2.3 Selection of the Study Subjects

T2DM subjects were selected from the Out-Patient Department, Bangladesh Institute of Research & Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) and Bangladesh Institute of Health Sciences hospital (BIHSH), and healthy control subjects were collected from the Bangladesh University of health sciences (BUHS), Dhaka WASA, SGS Ltd, University of Dhaka and Nilkhet Ladies Hostel respectively. This study was carried out on 371 type 2 diabetic subjects which were a new user of an oral hypoglycemic drug-like DPP-4 inhibitor, Biguanides, sulphonylureas, or others) and 326 non-diabetic healthy volunteers as control.

2.3.1 Inclusion Criteria

Patients' inclusion criteria were-

(i) Type 2 diabetes subjects: aged 30-60 years

(ii) HbA_1C value: 7-9%

(iii) Use of Oral Hypoglycemic Agent: DPP-4 inhibitor, metformin, sulphonylureas, or others

2.3.2 Exclusion Criteria

Exclusion criteria were-

(i) Evidence of hepatic dysfunction: ALT (SGPT) or AST (SGOT) >100 units

(ii) Evidence of renal dysfunction: S creatinine > 1.7mg/dl

(iii) Presence of malabsorption syndrome

(iv) Pregnant women

(v) Diabetic subjects are treated with insulin.

2.3.3 Questionnaire

Patients visit was scheduled prior to start the study. After collection of blood, baseline characteristics, including anthropometry and clinical examinations, were done. Socio-demographic data, family history, medical history was obtained by interviewing the participants on the first day of the visit using a pre-set questionnaire. The smoking status, including active and passive lifetime exposure to smoke of the participants, was also asked during the interview. According to WHO's Smoking and Tobacco Use Policy, a smoker smokes any tobacco product, either daily or occasionally. A daily smoker is someone who smokes any tobacco product at least once a day. An occasional smoker is someone who smokes, but not every day. A "tobacco user" is someone who uses any tobacco product (WHO 2008).

2.4 Anthropometric Measurements

2.4.1 Height (m)

Using an appropriate scale (Detecto-Medic; Detecto Scales Inc., USA), the standing height was measured without shoes. The subject was positioned fully erect, with the head in the Frankfurt plane (with the line connecting the outer canthus of the eyes and the external auditory meatus perpendicular to the long axis of the trunk); the back of the head, thoracic spine, buttocks, and heels touched the vertical axis of the anthrop meter, and the heels were together. Height was recorded to the nearest 5 millimeters.

2.4.2 Weight (kg)

The bodyweight of each participant was taken to the nearest 0.5 kg. The balance was placed on a hard flat surface and adjusted to zero before measurement. The weight of the subjects was measured barefoot, wearing light clothes.

2.4.3 Waist Measurement

This was done horizontally at the narrowest point between the lower end of the rib cage and the iliac crest in centimeters.

2.4.4 Hip Circumference

Hip circumference was measured at the greatest horizontal circumference below the iliac crust at the greater trochanter level in centimeters using a standard measuring tape.

2.4.5 Waist Hip Ratio (WHR)

Waist Hip Ratio was calculated using the standard formula. The optimal WC (WHR) cutoff values were 97–99 cm (0.95) for White men and 85 cm (0.83–0.85) for White women living outside the USA and the UK, whereas they were 85 cm (0.90) for Asian men and 75–80 cm (0.79–0.85) for Asian women; the values for other ethnic groups were between those for White and Asians (Qiao et al., 2010)

2.4.6 Total Body Fat Measurement

There are many specific techniques used for measuring body fat. The OMRON® Body Fat Analyzer used the Bioelectrical Impedance (BI) method to estimate body fat percentage. Body fat percentage refers to the body fat mass as part of the total body weight described as a percentage.

Body fat percentage (%) = (Body Fat Mass in kg / Body Weight in kg) x 100

2.4.7 BMI (kg/m^2)

The body mass index (BMI) of the subjects was calculated by using the following formula. *BMI= Weight (kg)/[height (m)]*$^{2.}$ Asian criteria-based BMI was used as follows: <18.5 for underweight, 18.5-22.9 for normal-weight, 23.0-27.5 for overweight, and >27.5 for obese women. International criteria-based BMI was used as follows: <18.5 for underweight, 18.5-24.9 for normal-weight, 25.0-29.9 for overweight, and ≥30 for obese women (WHO 2004)

2.4.8 Measurement of Blood Pressure

Blood pressure was measured in sitting position, with calf at the level of the heart. After 10 minutes of rest, a second reading was taken. Recorded Korot koff sound I (the first sound) and V (the disappearance of sound) denoted the systolic blood pressure (SBP) and diastolic blood pressure (DBP), respectively (according to WHO-IHS).

2.5 Blood Sample Collection

Blood samples from diabetic and healthy subjects were collected in the fasting condition. About 8 (eight) ml of venous blood was drawn from each participant by a trained person taking all aseptic precautions using a 10 ml disposable syringe. Approximately, four (4) ml blood was immediately transferred to a vacutainer tube for estimation of fasting glucose, lipid profiles, ALT, creatinine, and fasting insulin, and four (4) ml blood was preserved with EDTA-Na^2 containing tubes separately for HbA1C estimation and DNA extraction respectively; and kept in an icebox for transformation to the laboratory. Serum samples were separated from vacutainer tubes after centrifugation for 10 minutes at 3,000 rpm. Samples for HbA1c were estimated within 1 hour, and other samples (serum and whole blood) were stored at -20^0 and -80^0C until further use, respectively (**Figure 2.2)**.

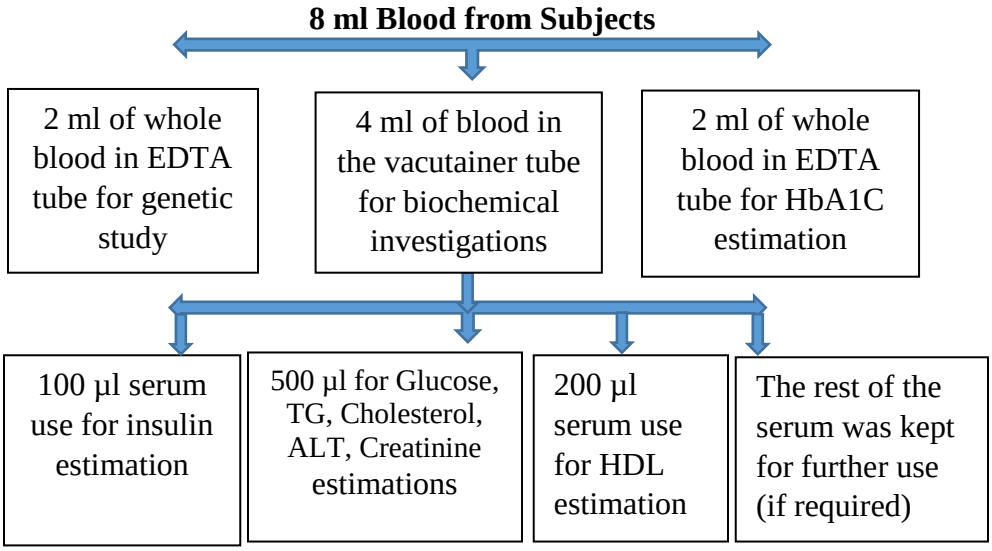


Figure 2.2: Flow Chart of Aliquot Distribution for Analysis

2.6 Biochemical Analysis

2.6.1 Estimation of Glucose

Serum glucose was estimated by enzymatic colorimetric (GOD-PAP) method using a commercial kit (Randox Laboratories, UK) (Barham and Trinder, 1972).

2.6.1.1 Principle

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under the catalysis of peroxidase, with phenol and 4-aminophenazone to create a red-violet quinoneimine dye as an indicator.

$$\text{Glucose} + H_2O \xrightarrow[\text{Oxidase}]{\text{Glucose}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4\ H_2O$$

2.6.1.2 Reagents

Table 2.1: Reagents for Glucose Estimation

Contents	**Initial Concentration of Solution**
Buffer	
Phosphate Buffer	0.1 mol/L, pH 7.0
Phenol	11 mol/L
GOD-PAP Reagent	
4-aminophenazone	0.77 mmol/L
Glucose oxidase	≥1.5 kU/L
Peroxidase	≥1.5 kU/L
Additional Reagent	Uranyl Acetate 0.16% (2 x 500 mL)
Standard	
Glucose	5.55 mmol/L (100 mg/dL)

2.6.1.3 Procedure

Glucose was estimated without deproteinization. The instrument was calibrated before estimation. Serum and reagent were taken in a specific cup. They were arranged serially into the Auto-Lab Analyzer (Analyzer Medical system, Rome, Italy). The Auto lab was programmed for the estimation of glucose and allowed to run with the following procedure:

5 µl sample and 500 µl reagent was mixed and incubated at 37° C for 10 minutes. The reaction occurred in a reaction cell. The absorbance of samples and standard was measured against the reagent blank at 500 nm within 60 minutes.

2.6.1.4 Calculation

Optical densities (OD) or absorbance were fed into a computer, and calculation was done using the software program. Values for the unknown samples were calculated by extrapolating the absorbance for the standard using the following formula:

$$\text{Glucose concentration (mmol/l)} = \frac{A_{Sample}}{A_{Standard}} \times 5.55$$

2.6.2 Estimation of Glycosylated Hemoglobin (HbA_{1c})

HbA_{1c} was estimated by BIO-RAD Kit (Mayer and Freedman, 1983).

2.6.2.1 Principle

The Bio-Rad VARIANT Hemoglobin A_{1c} program utilizes ion-exchange high-performance liquid chromatography (HPLC) principles for the automatic and accurate separation of HbA_{1c}. The separation of HbA_{1c} is performed rapidly and precisely, without interference from labile A_{1c}lipemia or temperature fluctuations. HbA1c, the glycohemoglobin of interest, is formed in two steps by the nonenzymatic glycation of HbA. The first step is forming an unstable aldimine (labile A1c, or pre-A1c), a reversible reaction between the carbonyl group of glucose and the N terminal valine of the β-chain of hemoglobin. Labile A1c formation is directly proportional to the blood glucose concentration. During red blood cell circulation, some of the labile A1c is converted

(Amadori rearrangement) to form a stable ketoamine, HbA1c. The D-10 Hemoglobin A1c Program is based on the chromatographic separation of HbA1c on a cation exchange cartridge. Separation is optimized to minimize interferences from hemoglobin variants, labile A1c, and carbamylated hemoglobin.

2.6.2.2 Reagents

Table 2.2: Reagents for Glycosylated Hemoglobin (HbA_{1c}) Estimation

Sample Vials	1000 polypropylene sample vials, 1.5 mL
Elution Buffer 1	2500 mL of sodium phosphate buffer, pH 5.9 contains <0.1% sodium azide as a preservative.
Elution Buffer 2	2000 mL of sodium phosphate buffer, pH 5.6 contains <0.1% sodium azide as a preservative.
Whole Blood Primer	Ten vials of lyophilized human red blood cell hemolysate with gentamicin, tobramycin and EDTA as preservatives.
Wash Solution	1800 mL deionized water with <0.05% sodium azide as a preservative.
Hemolysis Reagent	1100 mL of citrate solution contains <0.05% sodium azide as a preservative.
HbA_{1c} Calibrator	The calibrator contains lyophilized human red blood cell hemolysate with gentamicin, tobramycin, and EDTA as preservatives.
Analytic Cartridges	Two cation exchange cartridges.
ROM Card	Along with VARIANT HbA_{1c} program parameters.

Allow the Elution Buffers and Wash Solution to reach room temperature (15–30°C) before performing the assay. Mix each bottle by gently inverting prior to use.

2.6.2.3 Procedure

Allow sample tubes to reach room temperature (15–30 °C) before performing the assay and thoroughly mix the sample by gently inverting the tube. Preceding analysis, a simple preparation of the patient sample is required to hemolysis the blood and remove labile A_{1c}. Samples are first diluted with hemolysis reagent and then incubated at 18-28° C for a

minimum of 30 minutes. The VARIANT's two dual-piston pumps deliver a programmed buffer gradient of increasing ionic strength to the analytical cartridge. Prepared samples are automatically injected into the analytical cartridge, where the hemoglobin's are separated based on their ionic interactions with the material. The separated hemoglobin then passes through the filter photometer's flow cell, where changes in the absorbance (415 nm) are measured; an additional filter at 690 nm corrects background variations. A built-in integrator performs a reduction of raw data collected from each analysis. A calibrator is analyzed with each run for adjustment of the calculation parameters for the determination of $HbA_{1c.}$ A chromatogram of the changes in the absorbance is plotted versus the retention time. Each chromatogram printout is accompanied by a report identifying each peak detected, plus the relative percent and retention times of each peak.

To predilute, pipet 1.5 mL of Wash/Diluent Solution into a labeled 1.5 mL vial, followed by 5 µL of the whole blood sample. Cap the sample vial and mix thoroughly.

2.6.2.4 Calculation

The samples were run in the auto-analyzer. After the analysis of the calibrator, the calibration response factor for HbA_{1c} is automatically calculated. A sample report and a chromatogram are generated for each sample. The A1c peak is shaded. This area is calculated using an exponentially modified Gaussian (EMG) algorithm that excludes the labile A1c and carbamylated peak areas from the A1c peak area.

2.6.3 Estimation of Serum Insulin

The Insulin ELISA is an enzyme immunoassay for the quantitative *in vitro* diagnostic measurement of Insulin in serum and plasma (Starr et al., 1978).

2.6.3.1 Principle

The Insulin ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle. The microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on the insulin molecule. An aliquot of a patient sample containing endogenous insulin is incubated in the coated well with enzyme conjugate, an anti-insulin antibody conjugated with biotin. After incubation, the unbound

conjugate is washed off. During the second incubation step, Streptavidin Peroxidase Enzyme Complex binds to the biotin-anti-insulin antibody. The amount of bound HRP (Horseradish peroxidase enzyme) complex is proportional to insulin concentration in the sample. Having added the substrate solution, the intensity of color developed is proportional to insulin concentration in the subjects' sample.

2.6.3.2 Reagents

1. **Microtiter wells:** Wells were coated with anti-insulin antibody (monoclonal).
2. **Zero Standard:** Contains non-mercury preservative.
3. **Standard (Standard 1-5)**: Concentrations: 6.25, 12.5, 25, 50 and 100 μIU/mL, Conversion: μIU/mL x 0.0433 = ng/mL, ng/mL x 23.09 = μIU/mL
4. **Enzyme Conjugate**: Mouse monoclonal anti-insulin conjugated to biotin; Contains non-mercury preservative.
5. **Enzyme Complex:** Streptavidin-HRP Complex Contains non-mercury preservative.
6. **Substrate Solution:** Tetra-methyl-benzidine (TMB).
7. **Stop Solution:** contains 0.5 M H_2SO_4,
8. **Wash Solution:** 40X concentrated.

2.6.3.3 Wash Solution

Add deionized water to the 40X concentrated Wash Solution. Dilute 30 mL of concentrated Wash Solution with 1170 mL deionized water to a final volume of 1200 mL.

2.6.3.4 Procedure

Secure the desired number of Microtiter wells in the frame holder. Add 25 μL of each standard, control, and samples with new disposable tips into appropriate wells. Dispense 25 μL Enzyme Conjugate into each well. Thoroughly mix for 10 seconds for complete mixing in this step. Then incubate for 30 minutes at room temperature. After incubation, the plate was briskly shaken out of the contents of the wells. Rinse the wells three times with diluted Wash Solution (400 μL per well). Strike the wells sharply on absorbent paper to remove residual droplets for influencing the sensitivity and precision of this assay. Then

add 50 µL of Enzyme Complex to each well. Then, incubate for 30 minutes at room temperature. Again, briskly shake out the contents of the wells. Rinse the wells three times with diluted Wash Solution (400 µL per well). Strike the wells sharply on absorbent paper to remove residual droplets. Add 50 µL of Substrate Solution to each well. Again, incubate for 15 minutes at room temperature. Finally, the enzymatic reaction was stopped by adding 50 µL of stop solution to each well and then determined the absorbance (OD) of each well at 450 ± 10 nm with a microtiter plate reader. The plate was readout within 10 minutes after adding the Stop Solution.

2.6.3.5 Calculation

1. Calculate the average absorbance values for each set of standards, controls, and patient samples.
2. Using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration from the standard curve.
4. Automated method: The results in the Instructions for Use have been calculated automatically using a 4-Parametercurve fit. (4 Parameter Rodbard or 4 Parameter Marquardt are the preferred methods). Other data reduction functions may give slightly different results.
5. The concentration of the samples can be read directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted or reported as > 100 µIU/mL. For the calculation of the concentrations, this dilution factor has to be taken into account.

2.6.4 Calculation of HOMA B%, HOMA S%, and HOMA IR

HOMA B%, HOMA S%, and HOMA IR were calculated by HOMA-SIGMA software version 2.2.

2.6.5 Calculation of QUICKI and Secretory HOMA

QUICKI and Secretory HOMA were calculated by the following formula respectively-

QUICKI = {1 / (log(fasting insulin µU/mL) + log(fasting glucose mg/dL)}

Secretory HOMA= {fasting insulin (picomole) X 3.33 / (fasting glucose mmol/l-3.5)}.

2.6.6 Estimation of Total Cholesterol

Total cholesterol was measured by enzymatic endpoint method (cholesterol Oxidase/Peroxidase) in an auto-analyzer (Analyzer Medical System, Rome, Italy) using a reagent of Randox laboratories, UK (Trinder, 1988).

2.6.6.1 Principle

The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantiphyrine in the presence of phenol and peroxidase.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\textit{Cholesterol Esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\textit{Cholesterol Oxidase}} \text{Cholestene- 3-one} + H_2O_2$$

$$2\ H_2O_2 + \text{phenol} + \text{4-aminoantiphyrine} \xrightarrow{\textit{Peroxidase}} \text{quinoneimine} + H_2O$$

2.6.6.2 Reagents

Table 2.3: Reagents for Total Cholesterol Estimation

Contents (Reagents)	Initial Concentration of Solution
4-Aminoantipyrine	0.30 mmol/L
Phenol	6 mmol/L
Peroxidase	$\geq$ 0.5 U/mL
Cholesterol esterase	$\geq$ 0.15 U/mL
Cholesterol oxides	$\geq$ 0.1 U/mL
Pipes Buffer	80 mmol/L; pH 6.8
Standard	5.17 mmol/L (200 mg/dL)

2.6.6.3 Procedure

Serum and reagents were taken in a specific cell. They were arranged serially. Then ID number for each test was entered in the AUTOLAB. 5 μL sample and 500 μL reagent were mixed and incubated at 37^0C for 5 minutes within the Auto lab. The reaction occurred in a reaction cell or cup. The absorbance of the sample and the standard were measured against the reagent blank at 500 nm within 60 minutes.

2.6.6.4 Calculation

The concentration of cholesterol in the sample was calculated by using a software program with the following formula:

$$\text{Cholesterol concentration (mg/dL)} = \frac{A_{Sample}}{A_{Standard}} \times \text{concentration of standard.}$$

2.6.7 Estimation of Triglycerides

Serum triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in auto-analyzer (Analyzer Medical System, Rome, Italy) using a commercial kit of Randox laboratories, UK (Trinder, 1969).

2.6.7.1 Principle

The triglyceride is determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen- peroxide, 4- aminophenazone, and 4-chlorophenol under peroxidase's catalytic influence.

$$\text{Triglyceride} + H_2O \xrightarrow{Lipase} \text{glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow[Kinase]{Glycerol} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow[Oxidase]{Glycerol\ Phosphate} \text{dihydroxy acetone phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{4- chlorophenol} \xrightarrow{Peroxidase} \text{quinoneimine} + HCl + 4\ H_2O$$

2.6.7.2 Reagents

Table 2.4: Reagents for Triglycerides Estimation

Contents	Concentrations in the Test
Buffer	
Pipes Buffer	40 mmol/L, pH 7.6
4-choloro-phenol	5.5 mmol/L
Magnesium-ions	17.5 mmol/L
Enzyme Reagent	
4-aminophenazone	mmol/L
ATP	1.0 mmol/L
Lipases	>150 U/mL
Glycerol-3-phosphate oxidase	1.5 U/mL
Peroxidase	0.5 U/mL
Standard	2.29 mmol/L (200 mg/dl)

2.6.7.3 Procedure

Serum and reagents were taken in a specific cup or cell. They were arranged serially. Then ID number for each test was entered in the AUTOLAB. 5 µl sample and 500 µl reagent was mixed and incubated at 37 ^{0}C for 5 minutes within the AUTOLAB.

The reaction occurred in a reaction cell. The absorbance of the sample and the standard were measured against the reagent blank at 500 nm within 60 minutes.

2.6.7.4 Calculation

Triglyceride concentration was calculated by using a software program in AUTOLAB with the following formula.

$$\text{Triglyceride concentration (mg/dl)} = \frac{A_{Sample}}{A_{Standard}} \times \text{Concentration of standard.}$$

2.6.8 Estimation of High-Density Lipoprotein (HDL)-Cholesterol

2.6.8.1 Principle

High-Density Lipoproteins (HDL) was separated from chylomicrons, Very Low-Density Lipoproteins (VLDL), and Low-Density Lipoproteins (LDL) by the addition of a precipitating reagent (Phosphotungstic acid-magnesium chloride) to serum. After centrifugation, the cholesterol concentration in the HDL (high-density lipoprotein) fraction, which remains in the supernatant, was determined by the enzymatic colorimetric method using CHOD- PAP (Lopes-Verila et al., 1977).

2.6.8.2 Reagents

All reagents were purchased from RANDOX, UK, to determine the levels of high-density lipoprotein-cholesterol in serum samples.

Table 2.5: Reagents for HDL-Cholesterol Estimation

Content	Initial Concentration of Solution
Reagents (Precipitate)	
Phosphotungstic acid	0.55 mmol/L
Magnesium chloride	25 mmol/L
Buffered enzyme reagent	
Pipes buffer, pH 6.8	80 mmol/L
Phenol	6 mmol/L
Cholesterol oxidase	≥ 0.1 U/mL
Cholesterol esterase	≥ 0.15 U/mL
Peroxidase	≥ 0.5 U/mL
4-aminoantipyrine	0.3 mmol/L
Cholesterol Standard	50 mg/dl (1.29 mmol/L)

2.6.8.3 Procedure

In a centrifuge tube, samples (100 µL) and precipitating reagents (250 µL) were taken and homogenized by shaking in a vortex mixer. Then it was centrifuged at 4000 rpm for 10

minutes. The supernatant was used as a sample for a colorimetric assay. A series of the standard HDL cholesterol solution (0, 10, 20, 25, 30, 40, 45 mg/dL) were prepared by diluting the stock standard solution of HDL-Cholesterol (50 mg/dL). Standard HDL Cholesterol solutions (20 μL) of each concentration were taken in the initial six micro-well of the plate. The first two wells were kept blank. The serum (20 μL) was then taken in the remaining micro-wells of the plate, and the working reagent (200 μL) was added to all the wells. The mixture was then incubated for 5 minutes at 37 C, and the absorbance of the solution was measured at 490 nm with a micro-plate ELISA Reader (Bio-Tek ELx 808, USA).

Two parallel experiments were carried out for each sample. Thus, a calibration curve was obtained for the absorbance vs. concentrations of the standard solutions against a reagent blank. Based on the calibration curve, the unknown concentrations of cholesterol in the serum sample were measured, maintaining the same mixing and incubation conditions as for the standard solutions. The standard curve was drawn on every experimental day.

2.6.8.4 Calculation

The HDL-Cholesterol concentration of serum samples was determined by using the software program (kinetic-Cal) with the formula for micro-well plate ELISA Reader (Bio-Tek ELx 808, USA).

$$\text{HDL Cholesterol (mg/dl)} = \frac{\Delta A\ sample}{\Delta A\ standard} \times \text{Concentration of Standard}$$

2.6.9 Estimation of Low-Density Lipoprotein (LDL)-Cholesterol

The LDL-Cholesterol level was calculated by using the following Friedewald formula (Friedewald et al., 1972).

$$LDL\text{-}Cholesterol = Total\ cholesterol - (HDL\text{-}Cholesterol + \frac{1}{5} x\ Triglyceride)$$

2.6.10 Estimation of Creatinine

Estimation of creatinine in serum was done by alkaline-picrate methods (Beckman, 2001).

2.6.10.1 Principle

Creatinine in an alkaline solution reacts with picric acid to form a colored complex. The amount of the complex formed is directly proportional to the concentration of creatinine.

2.6.10.2 Reagents

Standard	177 umol/L (2 mg/dL)
Picric acid surfactant	35 mmol/L
Sodium hydroxide	0.32 mol/L

2.6.10.3 Preparation of Reagent

All reagents are supplied ready to use, stable to expiry date when stored at +15 to 25 ^{0}C.

2.6.10.4 Preparation of Working Reagent

Mix equal volumes of solutions 2+3, stable for 3 Days at + 15 to +25 ^{0}C.

2.6.10.5 Procedure

In 1.0 mL working reagent, 0.1 ml standard or 0.1 mL sample were added in specific cells, respectively. Then mix, and after 30 sec, the absorbance A1 of the standard and sample were taken in 492 nm wavelength. Exactly 2 min. Later again, take the absorbance A2 of the standard and sample, respectively.

2.6.10.6 Calculation

A2 - A1 = ΔA sample or ΔA standard

$$\text{Concentration of Creatinine (mg/dl)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times 100$$

2.6.11 Estimation of Alanine Amino Transferase (ALT)

ALT was estimated by UV method using ALT (GPT) Kit (Randox Ltd. UK) (IFCC, 1980).

2.6.11.1 Principle

$$\alpha\text{- oxogluterate + L – alanine} \xrightarrow{\text{ALT}} \text{L-glutamate + pyruvate}$$

$$\text{Pyruvate + NADH + } H^{+} \xrightarrow{\text{LD}} \text{lactate + } NAD^{+}$$

2.6.11.2 Reagents

Table 2.6: Reagents for Alanine Amino Transferase (ALT) Estimation

Contents	Concentration in the Test
1. Buffer/Substrate	
Tris buffer	100 mmol/L, pH 7.5
L-alanine	0.6 mol/L
2. **Enzyme/Coenzyme/α-oxoglutarate**	15 mmol/L
α-oxoglutarate	≥1.2 U/mL
LD NADH	0.18 mmol/L

2.6.11.3 Preparation of Solutions

1. Buffer/Substrate: Buffer/Substrate supplied in the kit was used as it is.
2. Enzyme/Coenzyme/α-oxoglutarate: One vial of Enzyme/Coenzyme/α-oxoglutarate 2 was reconstituted with the appropriate volume of Buffer/Substrate 1:

2 ml	for the	20 x	2 ml	kit (AL 1200)
10 ml	for the	20 x	10 ml	kit (AL 1205)
20 ml	for the	5 x	20 ml	kit (AL 1268)

One vial of Enzyme/Coenzyme/α-oxoglutarate two was reconstituted with a portion of Buffer/Substrate 1, and then the entire contents were transferred to bottle 1 rinsing bottle two several times.

2.6.11.4 Procedure

Hundred (100 µL) serum and 1 ml Enzyme/Coenzyme/α-oxo-glutarate were mixed well into the sample cup. Then took the initial absorbance (at 340 nm wavelength) after 1 minute. Again after 1, 2, and 3 minutes, the absorbance was taken. The absorbance change per minute was noted, and if the value is between 0.11 and 0.16 at 340 nm, only then the values for the first 2 minutes were used for the calculation.

2.6.11.5 Calculation

To calculate the ALT activity, the following formulae was used:

$$U/l = 1746 \times \Delta A\ 340\ nm/min$$

2.7. Genetic Analysis

2.7.1 DNA Extraction

A chemical method was used for DNA extraction (Bailes et al., 2007). In this method, less time is required. It's cost-effective and can yield high-quality DNA for Polymerase Chain Reaction (PCR) analysis.

2.7.2 Chemicals and Reagents for DNA Extraction

All chemicals and reagents are analytical grades.

2.7.2.1 EDTA (0.5 M), pH 8.0: 186.1 g of anhydrous EDTA (Merk; Lot no: K90595821) was added to 800 mL of distilled water. pH adjusted to 8.0 using a pH meter with NaOH pellets. Total volume made up to 1 liter with distilled water and autoclaved at 15 p.s.i. for 15 min.

2.7.2.2 1 M Tris-HC1, pH 7.6: 121.1 g of Tris base dissolved in 800 mL of distilled water. pH was adjusted with concentrated HCl. The mixture was allowed to cool to room temperature. Before finally correcting pH, solution made up to 1 liter with distilled water and autoclaved at 15 p.s.i. for 15 min.

2.7.2.3 Preparation of Red Blood Cell Lysis Buffer: 0.01 M Tris-HCl pH 7.6, 320 mM sucrose, 5 mM MgCl2, 1% Triton X 100 (Roth; Lot no: 292186639). 10 mL of 1 M

Tris, 109.54 g of sucrose, 1.01 g of MgCl2 was mixed and adjusted pH to 8.0 and finally added 10 mL of Triton X-100 to 800 mL of distilled water, and made up to 1 liter with distilled water and autoclaved at 15 p.s.i. for 10 min.

2.7.2.4 Preparation of Nucleic Lysis Buffer: 0.01 M Tris-HC1, 11.4 mM sodium citrate, 1 mM EDTA (Merk; Lot no: K90595821), 1% sodium dodecyl sulphate (SDS). 10 mL of 1 M Tris-HC1 (pH 7.6), 3.75 g of anhydrous EDTA (pH 8.0), 10 g SDS, 2.94 g of sodium citrate was taken and adjusted the pH to 8.0 and made up to 1 liter with distilled water and autoclaved 15 min at 15 p.s.i.

2.7.2.5 TE Buffer, pH 8.0: 5 ml of 1 M Tris-HCl, pH 7.6, 2 mL of 0.5 M EDTA was taken, and pH adjusted to 8.0 and made up to 1 liter with distilled water. pH adjusted to 8.0 and autoclave 15 min at 15 p.s.i.

2.7.2.6 Phenol, Chloroform, Iso-amyl Alcohol Reagent: Take 25 mL of Phenol (Merk; Lot no: 822296), 24 mL of Chloroform (Roth), and 1 mL of Iso-amyl alcohol in a bottle. Mix thoroughly and store at 4^0C.

2.7.2.7 Ethanol (100%) (Merk; Lot no: K36464686) prechilled to -20 ^{0}C.

2.7.3 Extraction Procedure

Before starting DNA extraction, the liquid blood sample was shaken gently by rotating the tube. Then, 500 µL of blood was poured into a 1.5 mL Eppendorf tube, and 1000 µL of red cell lysis buffer was added. The Microfuge tube was shaken gently (up to homogenizing), then span for 2 minutes at 7000 rpm in a centrifuge machine (Eppendorf centrifuge; 5415D). The supernatant was discarded, and repeat the procedure for two or three more times to remove hemoglobin. It was important to breakdown the pellet by vortexing and rinsing it well in red blood cell lysis buffer to clean the white blood cells from residual hemoglobin. The tube was placed on tissue paper for a few seconds downward, taking cross-contamination between different samples. Then, 400 µL of nucleic lysis buffer was added to the Eppendorf tube (if a pellet was formed during this step, the pellet was dissolved by pipetting). Then, 100 µL of saturated (5M) NaCl and 600 µL of Phenol, Chloroform, Iso-amyl alcohol reagent were added to the Eppendorf tube and mixed on a

vortex mixer at room temperature, then span it for 2 minutes at 7000 rpm. After that, 400 µL of supernatant was transferred to a new 1.5 ml Eppendorf tube. Then, 800 µL of cold (-20 ^{0}C) absolute ethanol was added and vortex gently. DNA appeared as a mucus-like strand in the solution phase. Then the microfuge tube was spin for one minute at 12000 rpm to precipitate. The supernatant was discarded carefully, and let the tube be completely dried at room temperature (Placing Eppendorf tube downward on the tissue paper). Then, 50 µL of TE was added to it and vortexed. The Eppendorf tube with DNA was kept in 4 ^{0}C or -20 ^{0}C for later uses.

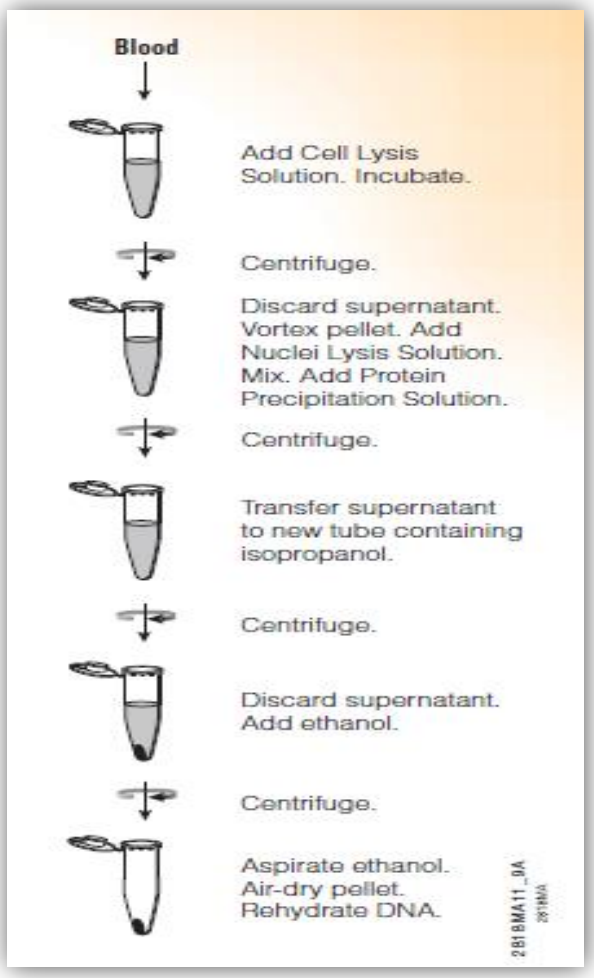


Figure 2.3: Steps of the DNA Extraction Procedure

We routinely used about 1 µL per PCR reaction without adverse effects. DNA can be quantified and diluted to a working concentration at this point or simply use 1 µL per PCR reaction.

2.7.4 Quantification of DNA

The quantity of the genomic DNA in the elute was determined by Nano-Drop 1000 spectrophotometer (NanoDrop 1000, US). The absorbance of diluted DNA was measured at 260 nm and 280 nm. The consistency of DNA elutes (5 µL) of the samples was also evaluated by agarose gel electrophoresis. A gel containing 0.5% agars was used for this purpose. The electrophoresis was performed at low voltage (40V) for an hour and visualized using a gel picture analyzer (AlphaImager Mini, USA).

2.8 PCR Amplification of the Target Genes Segment

The TCF7L2, SLC22A1, and KCNJ11 (E23K) genotypes were determined using the PCR-RFLP method.

2.8.1 Reagents

- Go StarTaq Polymerase (Promega, USA)
- dNTPs (New England Biolabs, USA)
- Primers (New England Biolabs, USA)
- DMSO (Merck)
- Restriction enzymes (Thermo scientific)
- Agarose (Sigma Chemical Co, USA)
- Ethidium bromide (Sigma Chemical Co, USA)
- 100 bp DNA ladder (Promega)
- TAE (Tris Acetate EDTA) Buffer

2.9 PCR of the KCNJ11 (E23K) Gene

Polymerase chain reaction (PCR) was carried out in a 14 µL reaction volume. PCR product size for the primer is 218 bp. PCR was carried out using the following set of primer:

rs5219	Primer
Forward primer	5´-GAATACGTCCTGACACGCCT-3´
Reverse primer	5´-GCCAGCTGCACAGGAAGGACAT-3´

PCR primer was constructed according to the protocol of Chidum et al. (2005). The primer sequences were verified using NCBI BLAST.

2.9.1 PCR Condition for KCNJ11 (E23K) Gene

PCR was carried out using GoTaq polymerase. Conditions for the amplification included the initial step of denaturation at 95 ^{o}C for 3 minutes followed by 35 cycles of denaturation at 95 ^{o}C for 30 seconds, annealing at 60 ^{o}C for 1 minute and elongation at 72 ^{o}C for 1 minute and finally a step of final elongation at 72 ^{o}C for 3 minutes. PCR assays were performed in a DNA thermal cycler (Applied Biosystems). PCR condition was fixed using the protocol of Chidum et al. (2005).

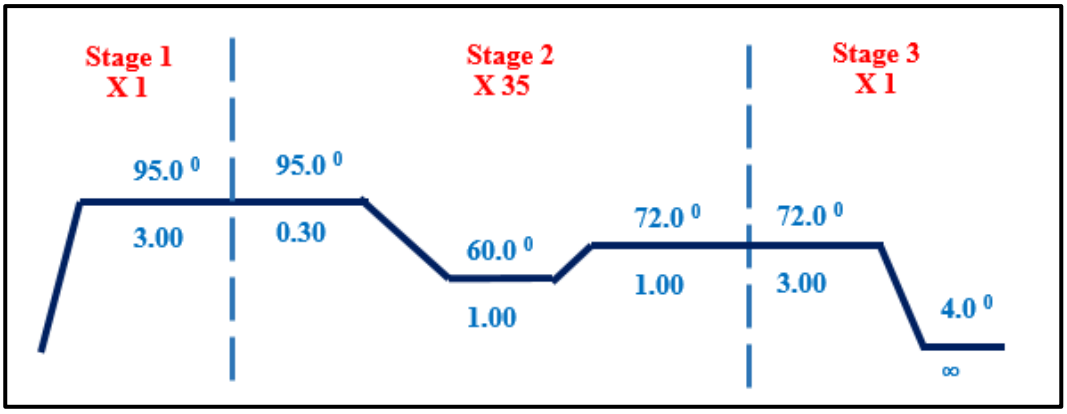


Figure 2.4: PCR Condition for KCNJ11 (E23K) Gene Amplification

2.9.2 Composition of PCR Product

Table 2.7 shows the composition of 14 µL reaction mixture for PCR.

Table 2.7 Composition of Reaction Mixture for KCNJ11-PCR

Name of the component	Volume (µL)
PCR-H_2O	6.8
5 X Green Buffer	3.0
DMSO	1.5
d NTPs	0.3
Reverse Primer	0.1
Forward Primer	0.1
Go Taq Polymerase	0.2
Genomic DNA	2.0
Total	**14 µL**

2.9.3 Evaluation of PCR

Five (5) µL of the PCR product was checked for amplification on a 2% agarose gel. The size of the product was ascertained by comparing it with 100 bp DNA ladder. The amplified DNA was stained with ethidium bromide solution and visualized under UV light (**Figure 3.7 and Figure 3.8**).

2.9.4 RFLP Analysis of KCNJ11 (E23K) Gene

The restriction enzyme Eco24I (BanII) was selected for PCR-RFLP studies using NEB cutter Version 2.0, an online tool for SNP-RFLP analysis. In this study, G>A (GAG>AAG or Lys23Gln) was used as a candidate marker. Eco24I (BanII) restriction enzyme recognizes GRGCY^C sites and cuts best at 37°C in the Tango buffer. Candidate gene analysis was carried out by PCR-RFLP method. Restriction enzyme digestion was performed following standard digestion protocol.

2.9.5 Eco 241 (Ban II) Restriction Digestion Protocol

PCR products were digested and incubated for 24 hours with Eco 241 (Ban II) at 37°C in a water bath. RFLP products were directly separated by electrophoresis in 3% agarose gel and visualized by ethidium bromide staining for 15 minutes. Eco 241 (Ban II) digestion produces three fragments depending on the genotype; E23E: 178 bp and 40 bp (Homozygous Wild type); K23K: 218 bp (Homozygous variants) and E23K: 218 bp, 178 bp and 40 bp (Heterozygous Variants). The digestion was carried out in a reaction volume of 15.5 μL. The enzyme digestion protocol was as follows:

Table 2.8: Composition of Reaction Mixture for Eco241 (BanII) Restriction Enzyme Digestion

Name of the Component	Volume (μL)
PCR reaction mixture	5.0
Nuclease free water	9.0
10 X Buffer	1.0
Eco241 (Ban II) RE	0.5
Total	**15.5**

2.10 PCR of the TCF7L2 Gene

Polymerase chain reaction (PCR) was carried out in a 15 μL reaction volume. PCR product size for the primer is 99 bp. PCR was carried out using the following set of primer:

rs12255372	Primer
Forward primer	5´-AGG AAT ATC CAG GCA AGC AT-3´
Reverse primer	5´-CTG GCA CTC AGAAGAGAGTCAG-3´

PCR primer was constructed according to the protocol of Shabnam et al. (2014). The primer sequences were verified using NCBI BLAST.

2.10.1 PCR Condition

PCR was carried out using GoTaq polymerase. Conditions for the amplification included an initial step of denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 55°C for 1 minute, and elongation at 72°C for 45 seconds and finally a step of final elongation at 72°C for 5 minutes. PCR assays were performed in a DNA thermal cycler (Applied Biosystems). PCR condition was fixed using the protocol of Shabnam et al. (2014).

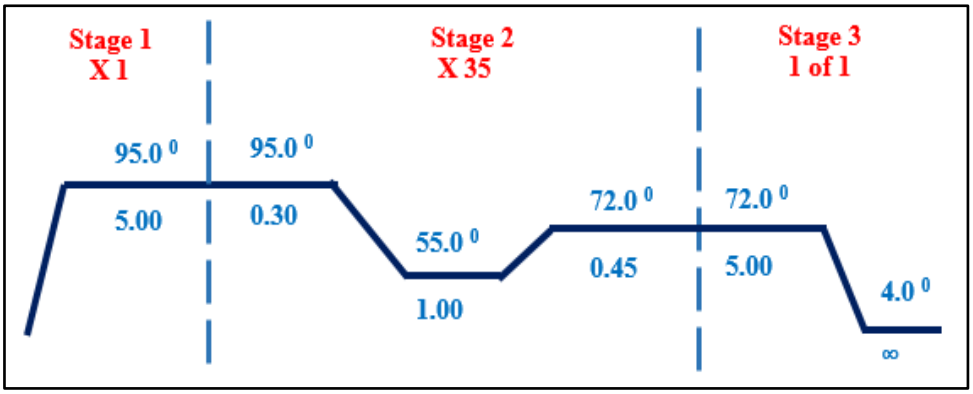


Figure 2.5: PCR Condition for TCF7L2 Gene Amplification

2.10.2 Composition of PCR Product

Table 2.10 shows the composition of 15 µL reaction mixture for PCR.

Table 2.9: Composition of Reaction Mixture for TCF7L2-PCR

Name of the Component	Volume (µL)
PCR-H_2O	7.4
5 X Green Buffer	3.0
DMSO	1.5
d NTPs	0.3
Reverse Primer	0.3
Forward Primer	0.3
Go Taq Polymerase	0.2
Genomic DNA	2.0
Total	**15 µL**

2.10.3 Evaluation of PCR

Five (5) µL of the PCR product was checked for amplification on a 2% agarose gel. The size of the product was ascertained by comparing it with 100 bp DNA ladder. The amplified DNA was stained with ethidium bromide solution and visualized under UV light.

2.10.4 RFLP Analysis of TCF7L2 Gene

The restriction enzyme Hin1II (Nla III) was selected for PCR-RFLP studies using NEB cutter Version 2.0, an online tool for SNP-RFLP analysis. In this study, G>T was used as a candidate marker. The 99 bp PCR product was subjected to restriction digestion using Hin1II (Nla III) following optimum reaction conditions as per manufacturer's protocol.

2.10.5 Hin1 II (Nla III) Restriction Digestion Protocol

PCR products were digested and incubated for 16 hours with Hin1 II (Nla III) at 37°C in a water bath. RFLP products were directly separated by electrophoresis in 3% agarose gel and visualized by ethidium bromide staining for 15 minutes. Hin1 II (Nla III) digestion produces three fragments depending on the genotype; GG: 78 and 21 bp (Homozygous Wild type); TT: 99 bp (Homozygous variants); and GT: 99, 78 and 21 bp (Heterozygous variants). The digestion was carried out in a reaction volume of 15.5 µL. The enzyme digestion protocol was as follows:

Table 2.10: Composition of Reaction Mixture for Hin1 II (Nla III) Restriction Enzyme Digestion

Name of the component	**Volume (µL)**
PCR reaction product	5.0
Nuclease free water	9.0
10 X Buffer	1.0
Hin1 II (Nla III) RE	0.5
Total reaction volume	**15.5 µL**

2.11 PCR of the SLC22A1 (OCT1) Gene

Polymerase chain reaction (PCR) was carried out in a 15.2 µL reaction volume. PCR product size for the primer is 422 bp. PCR was carried out using the following primer set:

rs628031	**Sequence**
Forward primer	5'-CTAAACCCAGTGATTCATGCTCTTT-3'
Reverse primer	5'-TTTGTTCTCATTCCAGAGGCTTATC-3'

PCR primer was constructed according to the protocol of Fazlollah et al. (2016). The primer sequences were verified using NCBI BLAST.

2.11.1 PCR Condition

PCR was carried out using GoTaq polymerase. Conditions for the amplification included an initial step of denaturation 95^{o}C for 5 minutes followed by 35 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 53^{o}C for 35 seconds, and elongation at 72^{o}C for 1 minute and finally a step of final elongation at 72^{o}C for 5 minutes. PCR assays were performed in a DNA thermal cycler (Applied Biosystems). PCR condition was fixed using the protocol of Fazlollah et al. (2016).

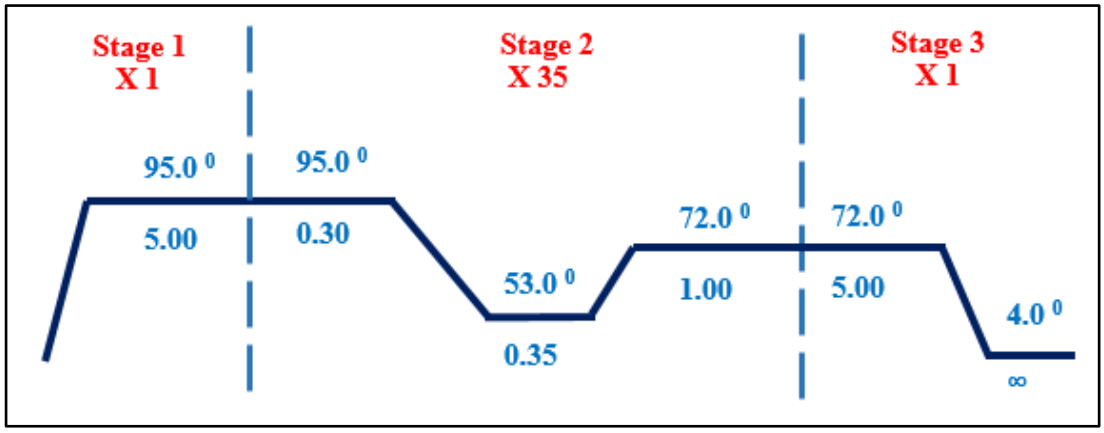


Figure 2.6: PCR Condition for SLC22A1 Gene Amplification

2.11.2 Composition of PCR Product

Table 2.1 shows the composition of 15 µL reaction mixture for PCR.

Table 2.11: Composition of Reaction Mixture for SLC22A1-PCR

Name of the component	Volume (µL)
PCR-H_2O	7.4
5 X Green Buffer	3.0
DMSO	1.5
d NTPs	0.3
Reverse Primer	0.3
Forward Primer	0.3
Go Taq Polymerase	0.2
Genomic DNA	2.0
Total	**15 µL**

2.11.3 Evaluation of PCR

Five (5) µL of the PCR product was checked for amplification on a 2% agarose gel. The size of the product was ascertained by comparing it with 100bp DNA ladder. The amplified DNA was stained with ethidium bromide solution and visualized under UV light.

2.11.4 RFLP Analysis of SLC22A2 Gene

The restriction enzyme MlsI (MscI) was selected for PCR-RFLP studies using NEB cutter Version 2.0, an online tool for SNP-RFLP analysis. MlsI (MscI) restriction enzyme recognizes TGG^CCA sites and cuts at 37°C. Candidate gene analysis was carried out by PCR-RFLP method. Restriction enzyme digestion was performed following standard digestion protocol.

2.11.5 MscI Restriction Digestion Protocol

PCR products were digested and incubated for 16 hours with MscI at 37°C in a water bath. RFLP products were directly separated by electrophoresis in 3% agarose gel and visualized by ethidium bromide staining for 15 minutes. MscI digestion produces three fragments depending on the genotype; GG: 154 bp and 268 bp (Homozygous Wild type); AA: 422 bp (Homozygous variants); and AG: 422 bp, 268 bp and 154 bp (Heterozygous variants). The digestion was carried out in reaction volume of 9.3 μL. The enzyme digestion protocol was as follows:

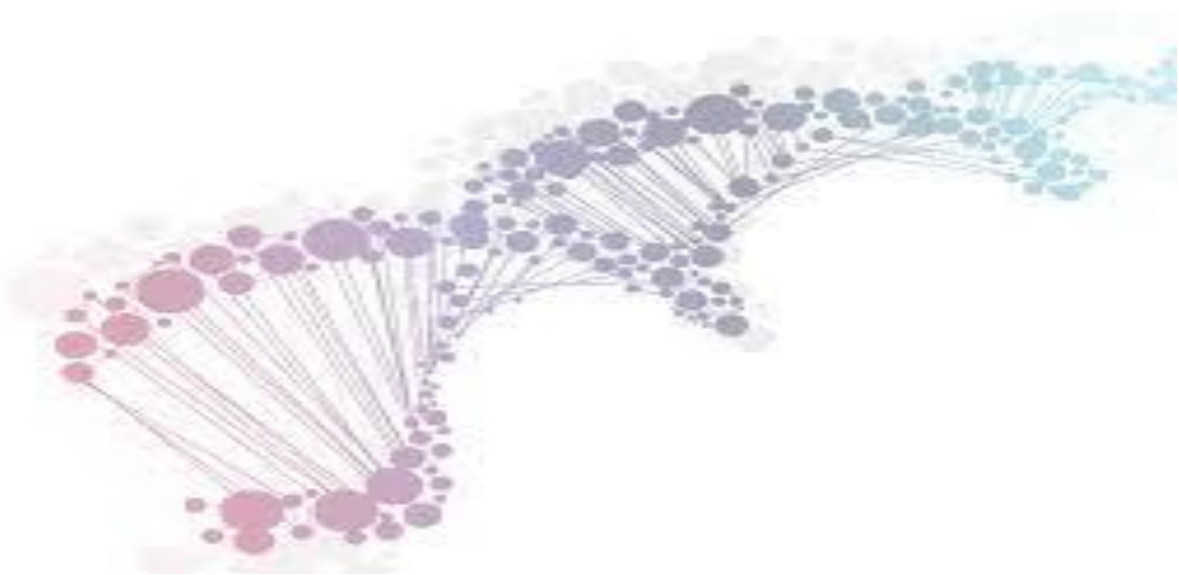
Table 2.12*:* Composition of Reaction Mixture for MscI Restriction Enzyme Digestion

Name of the Component	**Volume (μL)**
PCR reaction product	3.0
Nuclease free water	5.4
10X Buffer	0.6
MscI RE	0.3
Total	**9.3**

2.12 Statistical Analysis

The data were expressed as mean ± SD (Standard deviation). The statistical significance of differences between the values was assessed by univariate and multiple regression analysis, and one-way ANOVA was carried out using Statistical Package for Social Science (SPSS) version 22. The association of different biochemical and clinical parameters related to diabetes was also assessed after adjusting the relevant covariates of age, gender, and body weight. Statistical analysis was also performed using Graph Pad Prism version-6 software. The odds ratios (OR) were used as a measure of relative risk at 95% confidence intervals. Fisher's exact test was performed to analyze the association of respective gene polymorphisms with type 2 diabetes. *p*-value of <0.05 was considered statistically significant.

Chapter 3
Results



3. Results

3.1 Baseline Characteristics of the Study Subjects

3.1.1 Socio-Demographic Data of the Study Subjects

According to age, gender, marital status, area of residence, educational-, social-, occupational-, smoking status, and family history of diabetes, the baseline characteristics of study subjects are presented in **Table 3.1**. The study was carried out with 371 diabetic and 326 control subjects. The number of male subjects was higher in diabetic patients than that of control, 53% and 47%. The opposite phenomenon was found in female subjects, 47% and 53% for diabetic and control subjects, respectively. The age of diabetic and control subjects was 49±10 and 37±9 years, respectively. Among the control subjects, 70.9% were married, and 29.1% were unmarried, whereas in the diabetic group, 70.9% were married, and 29.1% were unmarried. According to area of residences, maximum subjects (85% of control and 61.2% of diabetic) were living in the urban area in comparison to the rural or semi-urban area; and there was no significant difference in the educational status of diabetic and control subjects, and the maximum number of subjects was noted; 54.9%, control, and 29.4%, diabetic were graduates or postgraduates. According to the income of the study subjects, the social status was divided into poor (Taka <5,000/month), lower middle class (Taka<15,000/month), upper-middle class (Taka <30,000/month), and rich (Taka>30,000/month) respectively. The proportion of control and diabetic subjects were 31.1% and 6.5% respectively in the poor; 42.0% and 48% in a lower middle class, 45.1% and 29.9% in upper-middle class; and 9.8% and 15.6 % in the rich group. The poor and upper-middle class were higher in diabetic patients compared to control, although not statistically significant. The occupational status of the subjects was divided into the unemployed and employed group. Among the study participants, 7.1% & 0.5% of the study subjects were students, 10.1% & 44.7% were housewives, 11% & 9.7% were jobless persons and 43.5% & 22.3% having job, 6.4% & 16.7% doing business and 21.8% & 5.9% were engaged in different profession in the control and diabetic subjects respectively. Smoking status was slightly higher in diabetic subjects than control (19.4% and 18.4%, respectively). The positive family history of diabetes was significantly ($p<0.001$) higher in diabetic subjects (69.5%) compared to the control (40.5%).

Table 3.1: Socio-Demographic Data of the Study Subjects

Variables		Study Subjects	
		Diabetic (n=371) (n, %)	**Control (n=326) (n, %)**
Gender			
Male		177 (47)	172 (53)
Female		194 (53)	154 (47)
Age (year)		49±10*	37±9
Marital status			
Married		364 (98.1)	231 (70.9)
Unmarried		7 (1.9)	95 (29.1)
Residence			
Rural		59 (15.9)	7 (2.1)
Semi Urban		85 (22.9)	42 (12.9)
Urban		227(61.2)	277 (85.0)
Educational status			
Undergraduate	*Illiterate*	45 (12.1)	13 (4.0)
	Read and Write	116 (31.3)	66 (20.2)
	SSC pass	44 (11.9)	33 (10.1)
	HSC pass	57 (15.4)	35 (10.7)
Graduate and Higher		109 (29.4)	179 (54.9)
Income/month (Social status)			
Poor (Tk.<5,000)		24 (6.5)	10 (31.1)
Lower middle (Tk.<15,000)		178 (48)	137 (42.0)
Upper middle (Tk.<30,000)		111 (29.9)	147 (45.1)
Rich (Tk.>30,000)		58 (15.6)	32 (9.8)
Occupational Status			
Unemployed	*Jobless*	36 (9.7)	36 (11.0)
	Student	2 (0.5)	23 (7.1)
	House Wife	166 (44.7)	33 (10.1)
Employed	*Professional*	83 (22.3)	142 (43.5)
	Business	62 (16.7)	21 (6.4)
	Technical	22 (5.9)	71 (21.8)
Smoking Status			
Non-Smoker		299 (80.6)	266 (81.6)
Smoker		72 (19.4)	60 (18.4)
Family History of Diabetes			
No		113 (30.5)	194 (59.5)
Yes		258 (69.5)*	132 (40.5)

Results are presented as mean ± standard deviation (SD). Data were compared using the Independent Sample T-Test. *p<0.001.

3.1.2 Anthropometric Data of the Study Subjects

Anthropometric measurements of the study subjects were shown in **Table 3.2**. The study subjects' weight and height were measured in kilograms and meter, respectively, to calculate the body mass index (BMI). BMI was 25.3±3.8 (Kg/m^2) in both case and control groups.

Waist and Hip were measured in centimeter to calculate the Waist-Hip Ratio (WHR). WHR was 0.9±0.2 and 0.9±0.1 between diabetic and control subjects, respectively, and showed a significant difference ($p<0.001$). Again, in the case of blood pressure, a significant ($p<0.001$) difference for both systolic blood pressure (121±12 mm Hg vs. 112±14 mm Hg) and diastolic blood pressure (79±7 mm Hg vs. 75±9 mm Hg) were found between diabetic and control subjects.

The fat monitor machine measured body fat mass, where the value was given in both percentage and kilogram. Fat percentage was significantly higher ($p<0.05$) in diabetic subjects (31±8) than that of the control (29±6).

Table 3.2: Anthropometric Data of the Study Subjects

Variables	Study Subjects		p value
	Diabetic (n=371)	**Control (n=326)**	
BMI (Kg/m^2)	25.3±3.8	25.3±3.8	*NS*
Waist:Hip ratio	0.9±0.2	0.9±0.1	***<0.001***
SBP (mmHg)	121±12	112±14	***<0.001***
DBP (mmHg)	79±7	75±9	***<0.001***
Body fat %	31±8	29±6	***<0.05***
Kg	20.5±8.0	19.4±6.1	*NS*

Results are presented as mean ± standard deviation (SD). Data were compared using the Independent sample T-Test. Differences were considered significant at $p<0.05$. *SBP: Systolic blood pressure; DBP: Diastolic blood pressure; BMI=Body Mass Index*

3.2 Clinical and Biochemical Data of the Study Subjects

Clinical and biochemical data for both diabetes and control were presented in **Table 3.3 and Table 3.4**.

Glycemic, insulinemic, alanine transferase (ALT), and creatinine data were shown in **Table 3.3**. Fasting blood glucose levels were 7.5±2.5 mM/L and 4.9±0.6 mM/L, and HbA1c% was 7.8±1.7 (%) and 5.1±0.5 (%) in diabetic and control subject, respectively. Both the glucose and HbA1c levels of diabetic subjects were significantly higher than that of control subjects ($p<0.001$). Fasting serum insulin level of diabetic subjects was significantly higher ($p<0.001$) than that of control subjects (23.8±14.2 µU/L vs. 14.5±5.8 µU/L respectively).

Table 3.3: Glycemic, Insulinemic, ALT and Creatinine Data of the Study Subjects

Variables	Study Subjects		*p value*
	Diabetic (n=371)	**Control (n=326)**	
Glucose (mM/L)	7.5±2.5	4.9±0.6	***<0.001***
HbA1c (%)	7.8±1.7	5.1±0.5	***<0.001***
Insulin (µU/L)	23.8±14.2	14.5±5.8	***<0.001***
GINR	0.41±0.29	0.44±0.32	*NS*
HOMA B%	117.7±79.7	149.0±55.9	***<0.001***
HOMA S%	41.5±24.8	69.3±46.7	***<0.001***
HOMA IR	3.2±1.8	1.8±0.7	***<0.001***
QUICKI	0.29±0.03	0.32±0.03	***<0.001***
Secretory HOMA	208.9±184.3	263.2±180.8	***<0.01***
ALT (U/L)	29±16	29±9	*NS*
Creatinine (mg/dL)	0.93±0.12	0.92±0.12	*NS*

Results are presented as mean ± standard deviation (SD). Groups of data were compared using the Independent Sample T-Test. Differences were considered significant at $p<0.05$. *GINR: Glucose insulin Ratio; HOMA B%= Beta Cell Function; HOMA S%= Beta Cell Sensitivity; HOMA-IR: Homeostasis Model of Assessment Insulin Resistance; QUICKI= Quantitative Insulin Sensitivity Check Index; Secretory HOMA: Secretory Homeostasis Model of Assessment, ALT= Alanine Transferase*

On the other hand, the HOMA B% (117.7±79.7 vs. 149.0±55.9; p<0.001); HOMA S% (41.5±24.8 vs. 69.3±46.7; p<0.001), QUICKI (0.29±0.03 vs. 0.32±0.03; p<0.001), and secretory HOMA (208.86±184.18 vs. 263.22±180.77; p<0.01) were significantly lower in diabetic subjects compared to control; whereas insulin resistance HOMA IR was significantly higher (3.2±1.8 vs. 1.8±0.7; p<0.001) in diabetic subjects compared to control. No significant differences were found in ALT and creatinine levels between the two groups.

Table 3.4 demonstrated the lipidemic status of the study subjects. Triglycerides level was significantly (p<0.01) higher in diabetic subjects (152±80 mg/dL) than that of control (133±47 mg/dL). Total cholesterol level was (166±53 mg/dL) and (171±33 mg/dL) in diabetic and control subjects respectively. Although the cholesterol level was lower in diabetic subjects than that of the control, the difference was not statistically significant. HDL level was significantly lower (p<0.001) in diabetic subjects (40±11 mg/dL) compared to control (51±14 mg/dL); and LDL level was significantly higher (p<0.001) in diabetic subjects (108±47 mg/dL) compared to control (95±33 mg/dL).

Table 3.4: Lipidemic Status of the Study Subjects

Variables	Study Subjects		p value
	Diabetic (n=371)	Control (n=326)	
Triglycerides (mg/dL)	152±80	133±47	***<0.01***
Total Cholesterol (mg/dL)	166±53	171±33	*NS*
HDL- Cholesterol (mg/dL)	40±11	51±14	***<0.001***
LDL- Cholesterol (mg/dL)	108±47	95±33	***<0.001***
TC: HDL-C	3.7±1.2	4.5±3.1	***<0.001***

Results are presented as mean ± standard deviation (SD). Groups of data were compared using the Independent Sample T-Test. Differences were considered significant at p<0.05. *HDL= High-Density Lipoproteins; LDL= Low-Density Lipoproteins, TC: HDL-C = Total Cholesterol and HDL- Cholesterol Ratio.*

3.2.1 Clinical and Biochemical Data of the Study Subjects according to the Family History of Diabetes

According to the family history of diabetes, glycemic, insulinemic, lipid profiles, ALT, and creatinine data were presented in **Table 3.5**. Glucose level was non-significantly higher in diabetic subjects with a positive family history of diabetes than the subjects without a family history of diabetes; whereas HOMA B%, HOMA S%, and Secretory HOMA were comparatively lower in the positive family history of diabetic subjects.

Table 3.5: Clinical and Biochemical Data of the Study Subjects according to the Family History of Diabetes

Variables	Study Subjects					
	Diabetic		*p value*	Control		*p value*
	YES (n=258)	NO (n=113)		YES (n=132)	NO (n=194)	
Glucose(mM/L)	7.6±2.7	7.3±2.2	*NS*	5.0±0.6	5.0±0.5	*NS*
HbA1c %	7.8±1.7	7.8±1.7	*NS*	5.2±0.5	5.1±0.5	***<0.05***
Insulin (µU/L)	23.7±14.2	24.2±14.3	*NS*	14.8±6.3	14.2±5.4	***<0.05***
HOMA B%	116.2±78.9	121.1±81.6	*NS*	152.9±63.1	146.3±50.5	*NS*
HOMA S%	41.1±23.5	42.3±27.7	*NS*	71.0±52.9	68.1±42.0	*NS*
HOMA IR	3.2±1.8	3.3±1.8	*NS*	1.9±0.8	1.8±0.7	***<0.05***
QUICKI	0.29±0.02	0.30±0.03	*NS*	0.33±0.03	0.33±0.03	*NS*
Secr HOMA	201.6±247.4	225.6±355.4	*NS*	279.9±220.6	251.9±147.3	***<0.05***
TG (mg/dL)	151±82	152±75	*NS*	131±52	135±44	***<0.05***
TC (mg/dL)	170±55	158±49	*NS*	172±33	171±32	*NS*
HDL (mg/dL)	39±10	42±11	*NS*	49±12	52±15	*NS*
LDL(mg/dL)	111±49	99±40	*NS*	96±33	94±32	*NS*
TC: HDL-C	4.80±3.51	3.89±1.36	*NS*	3.76±1.16	3.59±1.19	*NS*
ALT (U/L)	29±15	30±18	*NS*	28±8	30±10	*NS*
Creatinine (mg/dL)	0.9±0.1	0.9±0.1	*NS*	0.9±0.1	0.9±0.1	*NS*

Results are presented as mean ± standard deviation (SD). Groups of data were compared using the Independent Sample T-Test. Differences were considered significant at $p<0.05$. *HOMA B%= Beta Cell Function; HOMA S%= Beta Cell Sensitivity; HOMA IR: Homeostasis Model of Assessment Insulin Resistance; QUICKI= Quantitative insulin sensitivity check index; Secretory HOMA: Secretory Homeostasis Model of Assessment; TG= Triglycerides, TC=Total Cholesterol, HDL= High-Density Lipoproteins, LDL= Low-Density Lipoproteins, TC: HDL-C = Total Cholesterol and HDL- cholesterol ratio, ALT= Alanine Transferase*

Total cholesterol level was 170±55 mg/dL, LDL-cholesterol level 111±49 mg/dL, and cholesterol and HDL-C ratio were 4.80±3.51 in diabetic subjects with a positive family history of diabetes; whereas 158±49 mg/dL, 99±40 mg/dL, and 3.89±1.36 mg/dL respectively in diabetic subjects without a family history of diabetes. Significantly ($p<0.05$), a higher value was found for HbA1C, fasting insulin, HOMA IR, Secretory HOMA, and triglycerides level in control subjects with a positive family history of diabetes than that of without a family history of diabetes.

3.2.2 Clinical and Biochemical Data of the Diabetic Subjects according to HbA1C Status

Table 3.6 presented the glycemic, insulinemic, lipidemic, ALT, and creatinine data of the diabetic subjects according to their HbA1c status. According to the guideline of BIRDEM (Hajera et al., 2003), the diabetic study subjects were divided into two groups: Mild/Moderate group (HbA1C<8) and Severe group (HbA1C>8). Serum glucose level and GINR were significantly higher ($p<0.001$ and $p<0.05$) in a severe diabetic group compared to mild/moderate diabetic groups. On the other hand, HOMA B% and QUICKI were significantly lower ($p<0.001$ and $p<0.001$ respectively) in severe diabetic subjects compared to mild/moderate diabetic subjects.

Table 3.6: Clinical and Biochemical Data of the Diabetic Subjects according to HbA1c Status

Variables	HbA1c Status of Diabetic Subjects		*p value*
	Severe (n=136) (HbA1C>8)	**Mild/Moderate (n=235) (HbA1C<8)**	
Glucose (mM/L)	8.6±3.0	6.8±2.0	***<0.001***
HbA1C (%)	9.6±1.4	6.7±0.7	***<0.001***
Insulin (µU/L)	24.1±14.8	23.3±13.5	*NS*
GINR	0.46±0.32	0.39±0.27	***<0.05***
HOMA B%	96.2±70.5	129.9±82.8	***<0.001***
HOMA S%	40.1±22.6	42.5±25.9	*NS*
HOMA IR	3.3±1.9	3.1±1.7	*NS*
QUICKI	0.29±0.02	0.30±0.02	***<0.01***
Secr HOMA	155.9±163.9	239.1±332.2	*NS*
TG (mg/dL)	158±79	146±77	*NS*
TC (mg/dL)	167±62	165±47	*NS*
HDL (mg/dL)	40±12	40±9	*NS*
LDL (mg/dL)	108±52	107±44	*NS*
TC:HDL-C	4.43±1.97	4.58±3.53	*NS*
ALT (U/L)	31±10	28±16	*NS*
Creatinine (mg/dL)	0.9±0.1	0.9±0.1	*NS*

Results are presented as mean ± standard deviation (SD). Groups of data were compared using the Independent Sample T-Test. Differences were considered significant at $p<0.05$. *GINR: Glucose insulin Ratio; HOMA B%= Beta Cell Function; HOMA S%= Beta Cell Sensitivity; HOMA-IR: Homeostasis Model of Assessment Insulin Resistance; QUICKI= Quantitative insulin sensitivity check index; Secretory HOMA: Secretory Homeostasis Model of Assessment; TG== Triglycerides, TC=Total Cholesterol, HDL= High-Density Lipoproteins, and LDL= Low-Density Lipoproteins. TC: HDL-C = Total Cholesterol and HDL- Cholesterol Ratio, ALT= Alanine Transferase.*

3.2.3 Clinical and Biochemical Data of the Study Subjects according to the Educational Qualification

Glycemic, insulinemic, lipidemic, ALT, and creatinine data of the study subjects according to the educational qualification were presented in **Table 3.7**. Educational qualification was sub-divided into two groups: undergraduate and graduate (including higher/postgraduate).

As shown in **Table 3.7**, no significant differences were found in glucose and HbA1c% levels between undergraduate and graduate (& higher) diabetic subjects, but HbA1c% level showed significantly higher ($p<0.001$) value (5.2±0.5%) in undergraduate control subjects than graduate (& higher) control subjects (5.0±0.5%). On the other hand, the triglycerides level was significantly higher ($p<0.05$) in graduate (& higher) control subjects than undergraduate control subjects.

Fasting insulin value (27.1±17.1 μU/L vs 22.5±12.7; μU/L), HOMA B% (133.1±92.3 % vs 111.3±73.0 %) and insulin resistance value (3.6±2.2 vs 3.0±1.6) were significantly higher ($p<0.01$, $p<0.05$ and $p<0.01$ respectively); and HOMA S% (37.7±20.0% vs 43.0±26.5 %) and GINR (0.37±0.25 vs 0.43±0.31, $p<0.05$) value were lower in diabetic subjects with graduate and postgraduate education than undergraduate diabetic subjects respectively. LDL cholesterol level was significantly higher ($p<0.05$) in undergraduate diabetic subjects (111±50 mg/dL) than graduate (& higher) diabetic subjects (98±35 mg/dL).

On the other hand, fasting glucose level was significantly lower (5.0±0.5 mM/L vs. 5.2±0.5 mM/L, $p<0.001$) and triglyceride level was significantly higher (139±50 mg/dL vs. 126±41 mg/dL, $p<0.05$) in control subjects with graduate and postgraduate education than undergraduate control subjects.

Table 3.7: Clinical and Biochemical Data of the Study Subjects according to Educational Qualification

Variables	Study Subjects					
	Diabetic			Control		
	Undergraduate (n=262)	Graduate (& higher) (n=109)	*p value*	Undergraduate (n=147)	Graduate (& higher) (n=179)	*p value*
Glucose (mM/L)	7.5±2.5	7.3±2.6	*NS*	5.0±0.5	5.0±0.6	*NS*
Hba1C (%)	7.7±1.6	7.9±2.0	*NS*	5.2±0.5	5.0±0.5	***<0.001***
Insulin (µU/L)	22.5±12.7	27.1±17.1	***<0.01***	14.1±5.9	14.8±5.7	*NS*
GINR	0.43±0.31	0.37±0.25	***<0.05***	0.48±0.38	0.41±0.25	*NS*
HOMA B%	111.3±73.0	133.1±92.3	***<0.05***	143.7±54.2	153.3±57.3	*NS*
HOMA S%	43.0±26.5	37.7±20.0	*NS*	74.3±53.8	65.2±39.6	*NS*
HOMA IR	3.0±1.6	3.6±2.2	***<0.01***	1.8±0.7	1.9±0.8	*NS*
QUICKI	0.30±0.02	0.29±0.03	*NS*	0.33±0.03	0.32±0.02	*NS*
Secr HOMA	197.0±287.3	237.6±275.6	*NS*	245.2±155.2	278.0±198.5	*NS*
TG (mg/dL)	153±82	149±74	*NS*	126±41	139±50	***<0.05***
TC (mg/dL)	169±56	159±45	*NS*	169±32	174±33	*NS*
HDL (mg/dL)	40±10	40±12	*NS*	50±16	52±13	*NS*
LDL (mg/dL)	111±50	98±35	***<0.05***	92±32	97±34	*NS*
TC:HDL-C	4.62±3.39	4.29±1.99	*NS*	3.77±1.34	3.58±1.02	*NS*
ALT (U/L)	29±17	30±14	*NS*	29±8	29±10	*NS*
Creatinine (mg/dL)	0.9±0.1	0.9±0.1	*NS*	1.0±0.1	0.9±0.1	*NS*

Results are presented as mean ± standard deviation (SD). Groups of data were compared using the Independent sample T-Test. Differences were considered significant at $p<0.05$. *GINR: Glucose insulin Ratio; HOMA B%= Beta Cell Function; HOMA S%= Beta Cell Sensitivity; HOMA-IR: Homeostasis Model of Assessment Insulin Resistance; QUICKI= Quantitative insulin sensitivity check index; Secretory HOMA: Secretory Homeostasis Model of Assessment; TG= Triglycerides, TC=Total Cholesterol, HDL= High-Density Lipoproteins, LDL= Low-Density Lipoproteins. TC: HDL-C = Total Cholesterol: HDL- Cholesterol Ratio, ALT= Alanine Transferase.*

3.2.4 Clinical and Biochemical Data of the Study Subjects according to the Area of Residence

As shown in **Table 3.8**, the study subjects were divided into rural, semi-urban, and urban groups according to the subjects' area of residence.

Glycemic and insulinemic status were not significantly changed in diabetic subjects based on the area of residence. But, total cholesterol level was significantly higher ($p<0.05$) in diabetic subjects living in a semi-urban area (179±61 mg/dL) than that of rural (161±48 mg/dL) and urban (163±51 mg/dL) area respectively. The glucose level was significantly higher in control subjects living in semi-urban areas than the rural and urban areas. On the other hand, HOMA B% and secretory HOMA were significantly higher ($p<0.05$ both), and HOMA S% was significantly lower ($p<0.05$) in control subjects living in the rural area than the semi-urban and urban area. The total cholesterol level was significantly high ($p<0.05$) in control subjects living in an urban area than in other areas.

Table 3.8: Clinical and Biochemical Data of the Study Subjects according to the Area of Residence

Variables	Study Subjects							
	Diabetic (n=371)			*p value*	Control (n=326)			*p value*
	Rural (n=59)	Semi urban (n=85)	Urban (n=277)		Rural (n=7)	Semi urban (n=42)	Urban (n=277)	
Glucose (mM/L)	7.4±2.3	7.7±2.3	7.4±2.7	*NS*	4.9±0.6	5.0±0.5	5.0±0.6	*NS*
HbA1C (%)	7.8±1.5	7.9±1.6	7.7±1.8	*NS*	5.0±0.3	5.5±0.6	5.1±0.5	***<0.001***
Insulin (µU/L)	25.0±17.4	24.3±14.4	23.3±13.3	*NS*	14.6±6.6	12.8±6.0	14.7±5.7	*NS*
HOMA B%	115.0±77.1	110.7±73.0	121.0±82.7	*NS*	153.1±61.4	126.3±43.8	152.4±56.9	***<0.05***
HOMA S%	42.9±29.9	41.9±28.6	40.9±21.8	*NS*	64.3±29.4	86.5±62.6	66.8±43.8	***<0.05***
HOMA IR	3.2±2.0	3.2±1.7	3.2±1.7	*NS*	1.9±0.8	1.6±0.7	1.9±0.7	*NS*
QUICKI	0.29±0.03	0.29±0.03	0.30±0.03	*NS*	0.33±0.02	0.34±0.04	0.33±0.03	*NS*
Secr HOMA	207.1±259.1	195.7±206.7	214.3±314.8	*NS*	277.7±188.7	200.3±105.8	272.5±188.1	***<0.05***
TG (mg/dL)	150±74	165±94	147±75	*NS*	139±38	128±42	134±48	*NS*
TC (mg/dL)	161±48	179±61	163±51	***<0.05***	167±34	159±30	174±33	***<0.05***
HDL (mg/dL)	39±9	40±10	41±11	*NS*	52±14	49±15	51±14	*NS*
LDL (mg/dL)	105±38	123±52	103±45	*NS*	102±51	85±29	96±33	*NS*
ALT (U/L)	32±21	30±19	28±13	*NS*	28±6	30±7	29±10	*NS*
Creatinine (mg/dL)	0.9±0.1	0.9±0.1	0.9±0.1	*NS*	0.9±0.1	0.9±0.1	0.9±0.1	*NS*

Results are presented as mean ± standard deviation (SD). Groups of data were compared using ANOVA. Differences were considered significant at $p<0.05$. *GINR: Glucose insulin Ratio; HOMA B%= Beta Cell Function; HOMA S%= Beta Cell Sensitivity; HOMA-IR: Homeostasis Model of Assessment Insulin Resistance; QUICKI= Quantitative insulin sensitivity check index; Secretory HOMA: Secretory Homeostasis Model of Assessment; TG= Triglycerides, TC=Total Cholesterol, HDL= High-Density Lipoproteins, and LDL= Low-Density Lipoproteins, ALT= Alanine Transferase.*

3.3 Correlation among Clinical and Biochemical Parameters of the Diabetic Study Subjects

The correlation coefficient of fasting serum glucose with HbA1C, fasting insulin level, Insulin resistance (HOMA IR), triglycerides, total cholesterol, HDL and LDL cholesterol were r=0.378, r=0.169, r= 0.357, r=0.248, r=0.128 and r=0.242 in type 2 diabetic subjects respectively. This indicate that these were high degree of positive correlation with glucose among above variables in type 2 diabetic patient and the correlation were highly significant ($p<0.001$). In comparison with glucose, a significant ($p<0.001$) negative correlation was found with HOMA B% (r=-0.604), HOMA S% (r=-0.225), QUICKI (r=-0.560), Secretory HOMA (r=-0.390); but positive correlation was found with HbA1c (r=0.378), insulin (r=0.169), HOMA IR (0.357) and lipid profiles [TG (r=0.248), Cholesterol (r=0.284), HDL (r=0.128) and LDL (r=0.242)] respectively.

In comparison with the HbA1C level, a significant negative correlation was found with HOMA B%, Secretory HOMA, QUICKI, and a significant positive correlation were found with TG. A positive correlation coefficient of insulin was found with HOMA B% (r=0.457, $p<0.001$), HOMA IR (r=0.951, $p<0.001$), Secretory HOMA (r=0.363, $p<0.001$) and triglycerides level (r=0.161, $p<0.01$) and a negative correlation was found with HOMA S% (r=-0.689, $p<0.001$) and QUICKI (r=-0.809, $p<0.001$) respectively. The triglycerides level showed a positive correlation with total cholesterol (r=0.318, $p<0.001$) and LDL cholesterol level (r=0.215, $p<0.001$). Whereas, the total cholesterol was found to have a significant positive correlation (r=0.938, $p<0.001$), and HDL cholesterol was found to have a significant negative correlation coefficient with LDL cholesterol (r= -0.120, $p<0.05$) respectively (**Table 3.9**).

Table 3.9: Pearson's Correlation among Clinical and Biochemical parameters of the Diabetic Study Subjects

		Glucose	HbA1c	Insulin	HOMA B%	HOMA S%	HOMA IR	QUICKI	Secr HOMA	TG	ChoL	HDL	LDL	ALT	Creatinine
Glucose	**COE**	1													
	P value														
HbA1c	**COE**	**0.378****	1												
	P value	**<0.001**													
Insulin	**COE**	**0.169****	0.012	1											
	P value	**<0.001**	NS												
HOMA B%	**COE**	**-0.604****	**-0.237****	**0.457****	1										
	P value	**<0.001**	**<0.001**	**<0.001**											
HOMA S%	**COE**	**-0.225****	-0.060	**-0.689****	**-0.308****	1									
	P value	**<0.001**	NS	**<0.001**	**<0.001**										
HOMA IR	**COE**	**0.357****	0.081	**0.951****	**0.316****	**-0.743****	1								
	P value	**<0.001**	NS	**<0.001**	**<0.001**	**<0.001**									
QUICKI	**COE**	**-0.560****	**-0.172****	**-0.809****	-0.058	**0.857****	**-0.873****	1							
	P value	**<0.001**	**<0.001**	**<0.001**	NS	**<0.001**	**<0.001**								
Secr HOMA	**COE**	**-0.390****	**-0.152****	**0.363****	**0.814****	**-0.236****	**-0.251****	**-0.083****	1						
	P value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	NS							
TG	**COE**	**0.248****	**0.113***	**0.161****	**0.154****	**-0.143****	**0.198****	**-0.265****	**-0.128***	1					
	P value	**<0.001**	**<0.05**	**<0.01**	**<0.01**	NS	**<0.001**	**<0.001**	**<0.05**						
Chol	**COE**	**0.284****	0.008	0.146**	-0.120*	-0.085	**0.168****	**-0.228****	**-0.120***	**0.318****	1				
	P value	**<0.001**	NS	NS	NS	NS	**<0.01**	**<0.001**	**<0.05**	**<0.001**					
HDL	**COE**	**0.128***	-0.020	-0.064	**-0.123***	0.000	-0.017	-0.016	**-0.103***	-0.057	0.003	1			
	P value	**<0.05**	NS	NS	**<0.05**	NS	NS	NS	**<0.05**	NS	NS				
LDL	**COE**	**0.242****	-0.026	0.157**	-0.097	-0.095	0.167**	**-0.220****	-0.106	**0.215****	**0.938****	**-0.120***	1		
	P value	**<0.001**	NS	NS	NS	NS	**<0.01**	**<0.001**	NS	**<0.001**	**<0.001**	**<0.05**			
ALT	**COE**	**0.156****	0.099	**0.150****	-0.093	**-0.131***	0.141**	**-0.229****	-0.013	**0.153****	0.144**	**-0.104***	0.111	1	
	P value	**<0.01**	NS	**<0.01**	NS	**<0.05**	NS	**<0.001**	NS	**<0.001**	NS	**<0.05**	NS		
Creatinine	**COE**	**0.006**	-0.070	-0.054	-0.062	0.039	-0.047	0.038	-0.038	0.061	**0.152****	-0.034	0.090	**0.197****	1
	P value	**NS**	NS	NS	NS	NS	NS	NS	NS	NS	**0.01**	NS	NS	**<0.001**	

* Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.001 level (2-tailed).

HOMA B%= Beta Cell Function; HOMA S%= Beta Cell Sensitivity; HOMA-IR: Homeostasis Model of Assessment Insulin Resistance; QUICKI= Quantitative insulin sensitivity check index; Secretory HOMA: Secretory Homeostasis Model of Assessment; TG= Triglycerides, TC=Total Cholesterol, HDL= High-Density Lipoproteins, and LDL= Low-Density Lipoproteins, ALT= Alanine Transferase.

3.4 Regression Analysis of the Diabetic Study Subjects

3.4.1 Regression Analysis of Glucose with Insulin in Diabetic Study Subjects

Regression analysis of glucose level with insulin in diabetic subjects was shown in **Figure 3.1**. A significant positive correlation was found between fasting glucose and insulin levels in diabetic subjects ($p<0.001$).

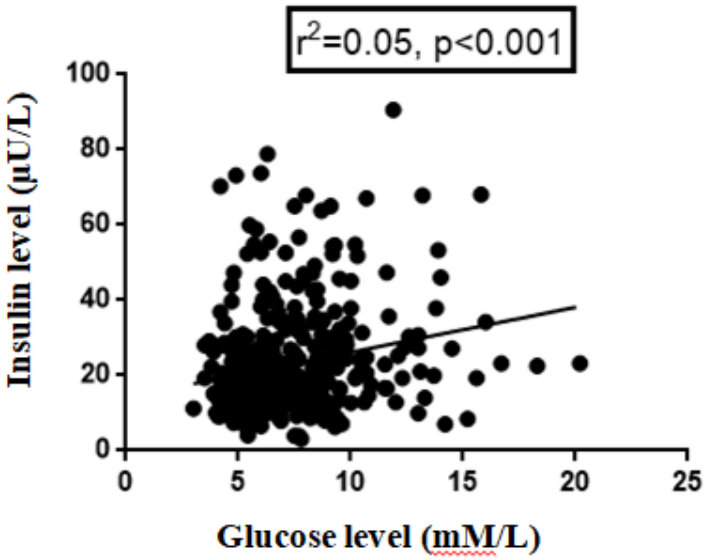


Figure 3.1: Regression Analysis of Glucose with Insulin in Diabetic Subjects

3.4.2 Regression Analysis of Glucose with HOMA B% and HOMA S% in Diabetic Study Subjects

Linear Regression analysis of fasting glucose with beta-cell function (HOMA B%) and insulin sensitivity (HOMA S%) in the T2DM subjects were presented in **Figure 3.2 and Figure 3.3**. A significant inverse association between fasting glucose with HOMA B% and HOMA S% was found ($p<0.001$), respectively.

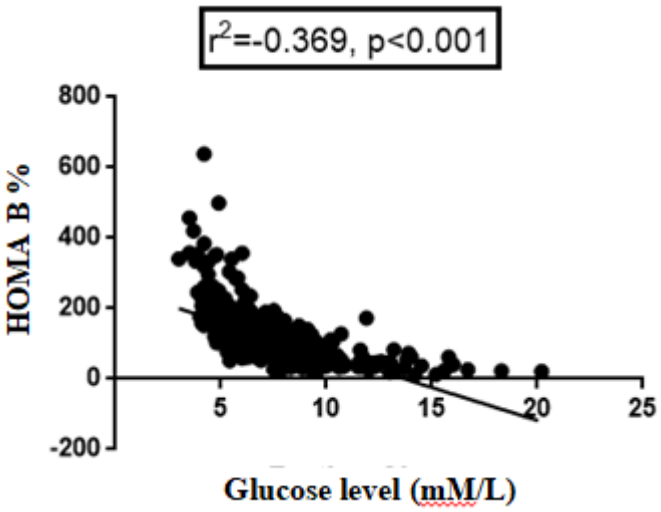


Figure 3.2: Regression Analysis of Glucose with Beta Cell Function (HOMA B%) in Diabetic Subjects

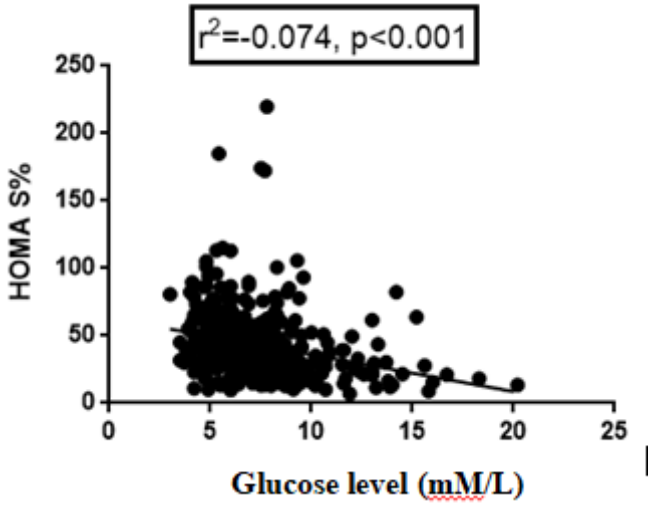


Figure 3.3: Regression Analysis of Glucose with Insulin Sensitivity (HOMA S%) in Diabetic Subjects

3.4.3 Regression Analysis of Glucose with HOMA IR, QUICKI, and Secretory HOMA in Diabetic Study Subjects

Figure 3.4, Figure 3.5, and **Figure 3.6** presented the regression analysis of glucose with insulin resistance (HOMA IR), QUICKI, and secretory HOMA in the T2DM subjects, respectively. A significant positive correlation between glucose with HOMA IR (r^2 = 0.159, p<0.001) and an inverse association between fasting serum glucose with QUICKI (r^2=-0.355, p<0.001) and secretory HOMA (r^2=-0.151, p<0.001) were found in type 2 diabetic subjects respectively.

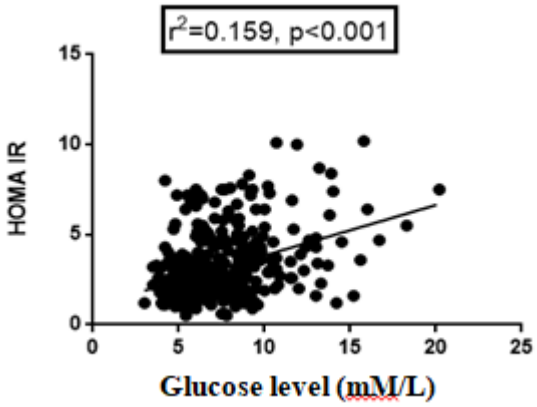


Figure 3.4: Regression Analysis of Glucose with HOMA IR in Diabetic Subjects

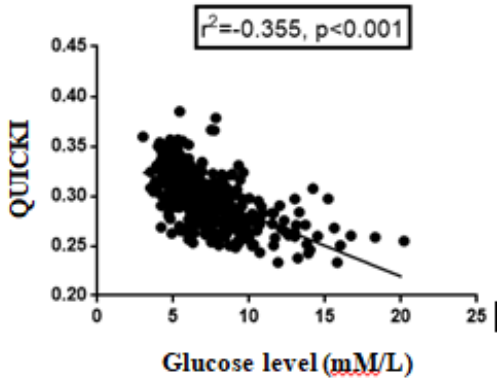


Figure 3.5: Regression Analysis of Glucose with QUICKI in Diabetic Subjects

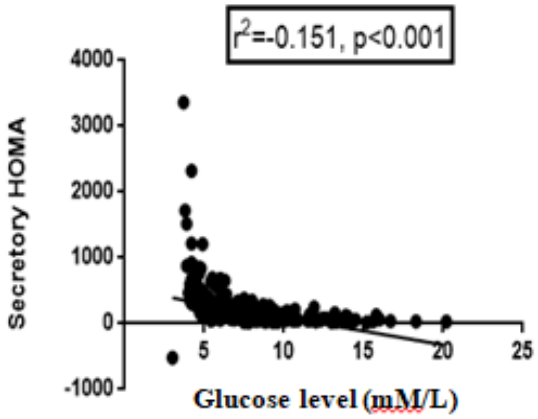


Figure 3.6: Regression Analysis of Glucose with Secretory HOMA in Diabetic Subjects

Study on
KCNJ11 Gene (E23K, rs5219)

3.5 Determination of KCNJ11 (E23K, rs5219) Gene Polymorphism

3.5.1 PCR Product for Detection of KCNJ11 (E23K) Gene Polymorphism

By using specific primers, a segment of the KCNJ11 gene was amplified, as shown in **Figure 3.7**. The length of the PCR product was 218 bp. The amplified PCR products (218 bp) were evaluated by 2% agarose gel electrophoresis, and the optional size of the PCR product was ascertained by comparing it with the 100bp DNA ladder.

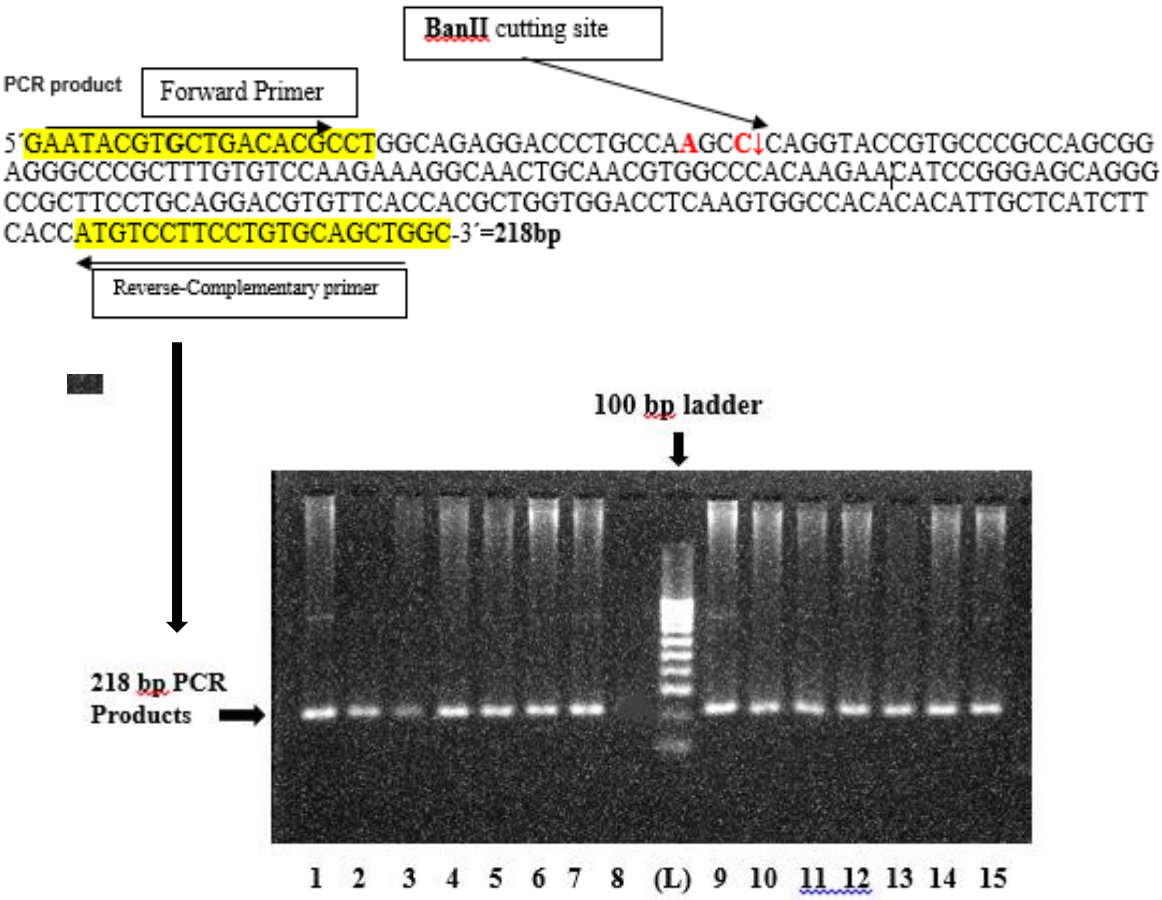


Figure 3.7: Representative PCR Products of KCNJ11 Gene in 2% Agarose Gel

- **Lane 1-7 and Lane 9-15:** show the PCR product of 218 bp, **Lane 8:** shows the absence of PCR, **Lane L**: 100 bp DNA ladder.

3.5.2 Restriction Digestion of the PCR Product of KCNJ11 Gene

To determine the KCNJ11 (E23K) genotype, amplified PCR products were digested with Eco24I (BanII) for 12 hours, and the final products were analyzed by electrophoresis in 3% agarose gel and visualized under UV light after EtBr staining. Mutation at codon 23 causes G>A (GAG>AAG) substitution at codon 23 that causes the replacement of glutamic acid by lysine (E23K). This mutation creates a restriction site of the Eco24I (BanII) restriction enzyme (RE). **Figure 3.8** represents the digestion products of the codon 23 allele by Eco24I (BanII) digestion. The complete digestion (218 bp and 178 bp) indicated that both polymorphic alleles were wild type (EE). Incomplete digestion with Eco24I (BanII) indicating heterozygote (Ht) mutant allele created 218 bp, 178 bp, and 40 bp fragment and absence of digestion with Eco24I (BanII), indicating homozygote (Hz) mutant allele created fragments of only 218 bp band (**Figure 3.8**).

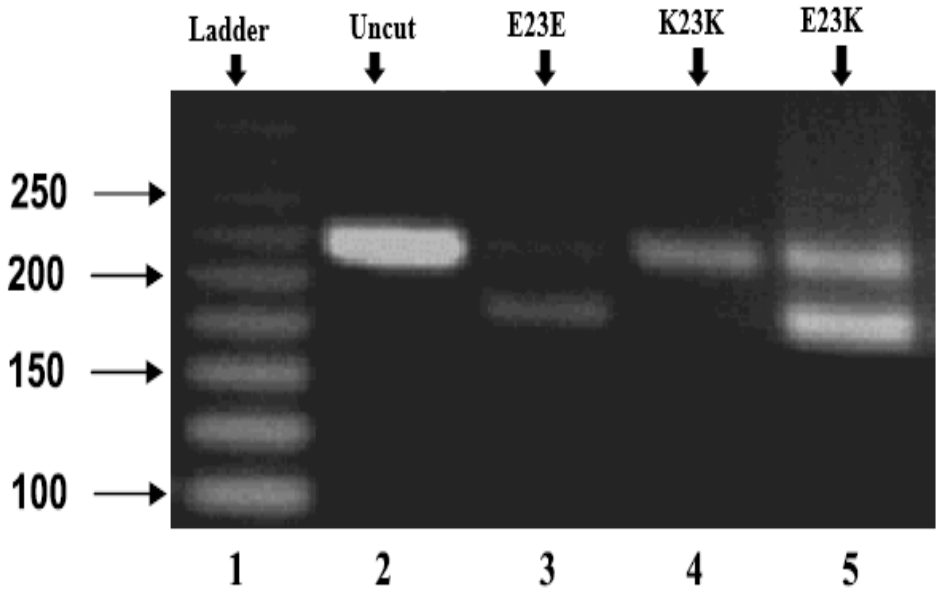


Figure 3.8: Representative Digested PCR Product of KCNJ11 (E23K) Gene

Lane 1: 100 bp DNA ladder, **Lane 2**: undigested sample 218 bp, **Lane 3:** Complete digestion with 178 and 40 bp, **Lane 4**: homozygous variants 218 bp, **Lane 5:** heterozygous variants 218, 178 and 40 bp. The 40 bp fragment was not shown.

3.5.3 Frequency Distribution of KCNJ11 Genotype and Risk of Diabetes

The distributions of KCNJ11 (E23K) genotype in type 2 diabetic and control subjects, and the estimated risk was presented in **Table 3.10**. As shown in **Table 3.10**, 53.37% of type 2 diabetic patients contained E23E, 42.86% contained E23K, and 3.77% contained K23K genotypes. Whereas 70.25% of the control subjects contained E23K, 27.91% contained E23K, and 1.84% had K23K genotypes, respectively. KCNJ11 variant of E and K allele frequency were 0.748 and 0.252 in diabetic and 0.842 and 0.158 in control subjects, respectively. Minor allele K was significantly high ($p<0.01$) in diabetic subjects than control subjects.

The frequency of the E23E, E23K, and K23K genotypes in patients with type 2 diabetes was compared to that of the control subjects. Significant associations were found for E23K ($p<0.001$), K23K ($p<0.05$), and E23K+K23K ($p<0.001$) when the E23E genotype was considered as the reference group **(Table 3.10)**.

Table 3.10: Genotypic Distribution and Diabetic Risk Assessment of KCNJ11 (E23K) Gene in the Study Subjects

	Study Subjects			
Genotype	**Diabetic (n=371) (n, %)**	**Control (n=326) (n, %)**	**OR (95% CI)**	***p value***
E23E	198 (53.37)	229 (70.25)	**1 (Reference)**	**---**
E23K	159 (42.86)	91 (27.91)	2.02 (1.47-2.78)	***<0.001***
K23K	14 (3.77)	6 (1.84)	2.70 (1.02-7.16)	***<0.05***
E23K+ K23K	173 (46.63)	97 (29.75)	2.06 (1.51-2.82)	***<0.001***
Allele	***(n, frequency)***			
E allele	277 (0.748)	274 (0.842)		
K allele	94 (0.252)	52 (0.158)		***<0.01***

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. $p<0.05$ was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). KCNJ11 genotypes, wild type homozygote, E23E; heterozygote variants, E23K; homozygote variants, K23K.

3.5.4 Frequency Distribution of KCNJ11 Genotype and Risk of Diabetes according to gender

The genotypic distribution of KCNJ11, according to gender, was presented in **Table 3.11**. A significant difference in the genotypic distribution of E23K was found in both diabetic males ($p<0.05$) and female ($p<0.001$) individually compared to the control subjects, when the E23E genotype was considered as the reference group. The result indicated that E23K variants might be a risk factor for both male and female diabetes subjects.

Table 3.11: Frequency Distribution of KCNJ11 (E23K) Genotypes in the Study Subjects according to gender

Gender	Genotype	Study Subjects		OR (95% CI)	*p value*
		Diabetic (n=371)	**Control (n=326)**		
		n=177 (n, %)	**n=172 (n, %)**		
Male	**E23E**	93 (52.5)	114 (66.3)	**1 (Reference)**	**---**
	E23K	76 (42.9)	53 (30.8)	1.75 (1.13-2.74)	***<0.05***
	K23K	8 (4.5)	5 (2.9)	1.96 (0.62-6.19)	*NS*
		n=194 (n, %)	**n=154 (n, %)**		
Female	**E23E**	105 (54.1)	115 (74.6)	**1 (Reference)**	**---**
	E23K	83 (42.8)	38 (24.7)	2.39 (1.50-3.81)	***<0.001***
	K23K	6 (3.1)	1 (0.6)	6.57 (0.78-55.5)	*NS*

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. $p<0.05$ was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). KCNJ11 genotypes, wild type homozygote, E23E; heterozygote variants, E23K; homozygote variants, K23K.

3.5.5 Frequency Distribution of KCNJ11 Genotype and Risk of Diabetes according to the Family History of Diabetes

The association between family history of diabetes and frequency of KCNJ11 genotype in diabetic and control subjects was presented in **Table 3.12.** According to **Table 3.12**, the frequency of E23K variants of KCNJ11 genotypes was significantly higher in diabetic subjects having a positive family history of diabetes compared to control [$p<0.001$, OR (95% CI) = 2.64 (1.65-4.23)]. No association was found in diabetic and control subjects

without a family history of diabetes. The result showed that the E23K genotype of KCNJ11 might be a high risk for diabetic subjects who have a positive family history of diabetes compared to without a family history of diabetes.

Table 3.12: Frequency Distribution of KCNJ11 Genotype with Risk of Diabetes according to the Family History of Diabetes

Family History of Diabetes	Genotype	Study Subjects		OR (95% CI)	*p value*
		Diabetic (n=371)	**Control (n=326)**		
		n=258 (n, %)	**n=132 (n, %)**		
YES	**E23E**	132 (51.1)	96 (72.8)	1 (Reference)	**---**
	E23K	116 (45.0)	32 (24.2)	2.64 (1.65-4.23)	***<0.001***
	K23K	10 (3.9)	4 (3.0)	1.82 (0.55-5.97)	*NS*
		n=113 (n, %)	**n=194 (n, %)**		
NO	**E23E**	66 (49.6)	133 (68.6)	1 (Reference)	**---**
	E23K	43 (32.3)	59 (30.4)	1.47 (0.90-2.40)	*NS*
	K23K	4 (3.0)	2 (1.0)	4.03 (0.72-2.58)	*NS*

Results are expressed as number (percentage). Fisher's exact test was performed to explore association between diabetic and control subjects for the genotype frequencies. $p<0.05$ was taken as level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). KCNJ11 genotypes, wild type homozygote, E23E; heterozygote variants, E23K; homozygote variants, K23K. *NS= Not Significant*

3.5.6 Influence of KCNJ11 Genotype on Risk of Diabetes with Age of Onset of Diabetes

Table 3.13 presented the risk of diabetes of KCNJ11 (E23K) genotypes according to the onset of diabetes in a different age group. The onset of diabetes was divided into three groups based on the age of the diabetic subjects: below 40 years, 40-50 years, and above 50 years, respectively. None of the variants were found significantly associated with diabetic risk in the case of E23K and K23K genotypes. The result suggested that genotypic variants of KCNJ11 may not be associated with age, indicating that the incidence of diabetes can occur in any stage of life.

Table 3.13: Influence of KCNJ11 Genotype on Risk of Diabetes with Age of Onset of Diabetes

Age (years) for Onset of diabetes	Genotype		OR (95% CI)
	E23K + K23K (n=173) (n, %)	E23E (n=198) (n, %)	
<40 (n=111)	56 (32.4)	55 (27.8)	**1 (Reference)**
41-50 (n=150)	66 (38.2)	84 (42.4)	0.77 (0.47-1.26)
>50 (n=110)	51 (29.4)	59 (29.8)	0.85 (0.50-1.44)

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. $p<0.05$ was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). KCNJ11 genotypes, wild type homozygote, E23E; heterozygote variants, E23K; homozygote variants, K23K.

3.5.7 KCNJ11 Genotype on Risk of Diabetes according to HbA1C Status

Association of KCNJ11 genotypes on the risk of diabetes, according to their HbA1C status, was presented in **Table 3.14**. As shown in **Table 3.14**, no significant association was found between the severity of diabetic according to HbA1C level with KCNJ11 variants E23K and K23K.

Table 3.14: KCNJ11 Genotype on Risk of Diabetes according to HbA1C Status

Genotype	HbA1C Status of Diabetic Subjects		OR (95% CI)
	Severe (HbA1C>8) (n=136) (n, %)	Mild/Moderate (HbA1C<8) (n=235) (n, %)	
E23E	72 (52.9)	126 (53.6)	**1 (Reference)**
E23K	59 (43.4)	100 (42.6)	1.03 (0.67-1.59)
K23K	5 (3.7)	9 (3.8)	0.97 (0.31-3.01)

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. $p<0.05$ was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). KCNJ11 genotypes, wild type homozygote, E23E; heterozygote variants, E23K; homozygote variants, K23K.

3.5.8 KCNJ11 Genotype on Risk of Diabetes according to the Smoking Status

Table 3.15 presented the frequency of KCNJ11 genotype in smoker and non-smoker of diabetic and control male subjects. Table 3.16 indicated that the E23K genotype frequency was significantly higher ($p<0.05$; OR=1.789; 95% CI=1.017-3.145) in non-smoker diabetic subjects compared to the control. No association of smoking status with the diabetic risk was found in E23K and K23K genotypes.

Table 3.15: KCNJ11 Genotype on Risk of Diabetes according to the Smoking Status

Smoking Status	Genotype	Study Subjects		OR (95% CI)	*P Value*
		Diabetic (n=177)	**Control (n=172)**		
		n=72 (%)	**n=55 (%)**		
Smoker	**E23E**	34 (47)	33 (60)	**1 (Reference)**	**---**
	E23K	33 (46)	20 (36)	1.601 (0.769-3.335)	*NS*
	K23K	5 (7)	2 (4)	2.426 (0.439-13.40)	*NS*
		n=105 (%)	**n=117 (%)**		
Non-Smoker	**E23E**	59 (56)	81 (69)	**1 (Reference)**	**---**
	E23K	43 (41)	33 (28)	1.789 (1.017-3.145)	***<0.05***
	K23K	3 (3)	3 (3)	1.373 (0.268-7.046)	*NS*

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. $p<0.05$ was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). KCNJ11 genotypes, wild type homozygote, E23E; heterozygote variants, E23K; homozygote variants, K23K. *NS= Not Significant.*

3.5.9 KCNJ11 Genotype on Risk of Diabetes according to the area of Residence

KCNJ11 Genotypes on the risk of diabetes, according to the area of residence between diabetic and control subjects was presented in **Table 3.16.** No association was found for E23K and K23K variants of KCNJ11 genotypes with residents living in the rural area. Whereas, E23K variant was found to have a significant association in semi-urban [p<0.05, OR (95% CI); 3.37 (1.26-8.93)] and urban [p<0.001, OR (95% CI); 2.14 (1.48-3.09)] residence diabetic subjects than control subjects respectively. The result indicated that the E23K genotype of KCNJ11 might be a diabetic risk factor for the residence of semi urban and urban subjects.

Table 3.16: KCNJ11 Genotype on Risk of Diabetes according to the Area of Residence

Area of Residence	Genotype	Study Subjects		OR (95% CI)	*p value*
		Diabetic (n=371)	**Control (n=326)**		
		n=59 (%)	**n=7 (%)**		
Rural	**E23E**	34 (58)	6 (86)	**1 (Reference)**	**---**
	E23K	22 (37)	1 (14)	3.88(0.437-34.50)	*NS*
	K23K	3 (5)	0 (0)	1.31 (0.06-28.70)	*NS*
		n=85 (%)	**n=42 (%)**		
Semi Urban	**E23E**	52 (62)	35 (83)	**1 (Reference)**	**---**
	E23K	30 (35)	6 (14)	3.37 (1.26-8.93)	***<0.05***
	K23K	3 (3)	1 (3)	2.02 (0.20-20.22)	*NS*
		n=227 (%)	**n=177 (%)**		
Urban	**E23E**	112 (49)	88 (50)	**1 (Reference)**	**---**
	E23K	107 (47)	84 (47)	2.14 (1.48-3.09)	***<0.001***
	K23K	8 (4)	5 (3)	2.68 (0.86-8.41)	*NS*

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. p<0.05 was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). KCNJ11 genotypes, wild type homozygote, E23E; heterozygote variants, E23K; homozygote variants, K23K. *NS= Not Significant.*

3.5.10 Distributions of Glycemic and Insulinemic Status according to KCNJ11 Genotype in the Study Subjects

According to genotype, analysis of glycemic and insulinemic status between control and diabetic subjects was presented in **Table 3.17.**

For diabetic subjects, glucose was higher in the K23K genotype (8.2±2.4) than the E23E (7.4±2.4) and E23K (7.5±2.7) genotypes non-significantly. Beta-cell function was non-significantly decreased in K23K variants (E23E vs. K23K, HOMA B%; 119.8±77.9 vs. 95.2±76.8) and E23K (E23E vs. E23K, HOMA B%; 117.1±82.2 vs. 95.2±76.8) variants in compared to E23E. The values of secretory HOMA for E23E, E23K, and K23K genotypes were 211.9±293.8, 209.1±278.6, and 162.9±212.5, respectively in diabetic subjects, although these values were not statistically significant (**Table 3.17**).

3.5.11 Distributions of Lipidemic, ALT, and Creatinine Status according to KCNJ11 Genotype in the Study Subjects

Genotypic distribution of lipidemic, ALT, and Creatinine status among study subjects was presented in **Table 3.18**. The serum triglycerides, total cholesterol, and LDL cholesterol levels were non-significantly higher. The HDL-cholesterol level was non-significantly lower in the homozygous genotype (K23K) than E23E homozygotes and E23K heterozygous genotype, respectively.

There were no significant variations found in serum ALT level based on genotypes both in case and control subjects. Although all serum creatinine values are within a normal range, the value was significantly higher in E23E homozygotes in diabetic subjects, whereas no differences were found in control subjects.

Table 3.17: Distribution of Glycemic and Insulinemic Status according to KCNJ11 Genotype in the Study Subjects

	Study Subjects							
	Diabetic (n=371)			***p value***	***Control (n=326)***			***p value***
Variables	**E23E *(n=198)***	**E23K *(n=159)***	***K23K* *(n=14)***		**E23E *(n=229)***	**E23K *(n=91)***	***K23K* *(n=6)***	
Glucose (mM/L)	7.4±2.4	7.5±2.7	8.2±2.4	*NS*	5.0±0.6	4.9±0.6	4.9±0.3	*NS*
HbA1c (%)	7.7±1.7	7.9±1.7	7.8±2.0	*NS*	5.1±0.5	5.1±0.5	5.2±0.3	*NS*
Insulin (µU/L)	24.1±14.7	23.7±13.9	21.5±10.6	*NS*	14.5±5.7	14.4±6.1	14.6±3.9	*NS*
GINR	0.41±0.31	0.41±0.27	0.47±0.23	*NS*	0.44±0.30	0.46±0.38	0.36±0.12	*NS*
HOMA B%	119.8±77.9	117.1±82.2	95.2±76.8	*NS*	148.2±54.2	150.9±61.9	151.4±24.6	*NS*
HOMA S%	41.1±25.1	41.8±24.7	42.1±24.8	*NS*	68.2±45.6	72.5±50.5	58.6±23.4	*NS*
HOMA IR	3.2±1.8	3.2±1.8	3.0±1.4	*NS*	1.8±0.7	1.8±0.7	1.9±0.5	*NS*
QUICKI	0.30±0.02	0.30±0.03	0.29±0.02	*NS*	0.33±0.03	0.33±0.03	0.33±0.02	*NS*
Secretory HOMA	211.9±293.8	209.1±278.6	162.9±212.5	*NS*	266.3±191.3	256.7±157.5	242.1±53.7	*NS*

Results are presented as mean ± standard deviation (SD). Groups of data were compared using a one-way ANOVA test. Differences were considered significant at $p<0.05$. KCNJ11 genotypes, wild type homozygote, E23E; heterozygote variants, E23K; homozygote variants, K23K. *GINR: Glucose insulin Ratio; HOMA B%= Beta Cell Function; HOMA S%= Beta Cell Sensitivity; HOMA-IR: Homeostasis Model of Assessment - Insulin Resistance; QUICKI= Quantitative Insulin Sensitivity Check Index; Secretory HOMA: Secretory Homeostasis Model of Assessment. NS=Not Significant.*

Table 3.18: Distributions of Lipidemic, ALT and Creatinine Status according to KCNJ11 Genotypes in the Study Subjects

Variables	*Study Subjects*							
	Diabetic (n=371)			*p value*	*Control (n=326)*			*p value*
	E23E *(n=198)*	**E23K *(n=159)***	***K23K* *(n=14)***		**E23E *(n=229)***	**E23K *(n=91)***	***K23K* *(n=6)***	
Triglycerides (mg/dL)	150±83	150±72	194±109	*NS*	134±46	131±48	121±36	*NS*
Total cholesterol (mg/dL)	168±55	163±53	170±40	*NS*	170±33	176±32	172±22	*NS*
HDL-Cholesterol (mg/dl)	40±10	41±11	34±7	*NS*	51±14	52±15	46±11	*NS*
LDL-cholesterol (mg/dL)	109±47	104±47	125±27	*NS*	94±32	96±34	96±37	*NS*
TC: HDL-C	4.6±3.5	4.4±2.5	5.1±1.6	*NS*	3.6±1.2	3.6±1.2	4.1±1.2	*NS*
ALT (U/L)	30±17	29±16	28±12	*NS*	29±9	30±9	32±5	*NS*
Creatinine (mg/dL)	0.94±0.12	0.90±0.11	0.92±0.12	*<0.05*	0.94±0.12	0.95±0.11	0.92±0.10	*NS*

Results are presented as mean ± standard deviation (SD). Groups of data were compared using a one-way ANOVA test. Differences were considered significant at $p<0.05$. KCNJ11 genotypes, wild type homozygote, E23E; heterozygote variants, E23K; homozygote variants, K23K. ALT= Alanine Amino Transferase, *TC: HDL-C=Total cholesterol: HDL-Cholesterol ratio, NS= Not Significant.*

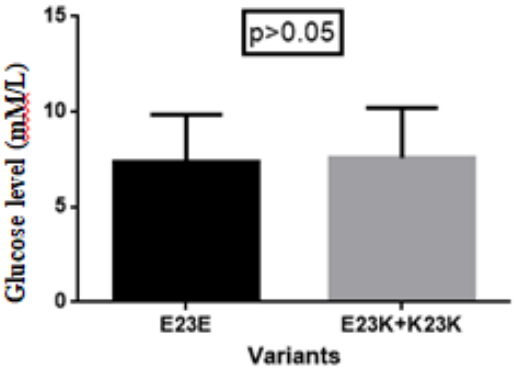


Figure 3.9: Glucose Level of Diabetic Study Subjects according to KCNJ11 Genotypes

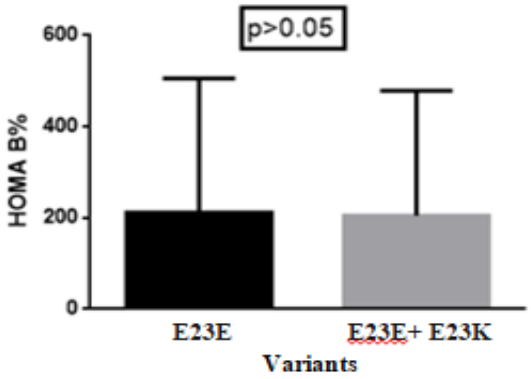


Figure 3.10: HOMA B% of Diabetic Study Subjects according to KCNJ11 Genotypes

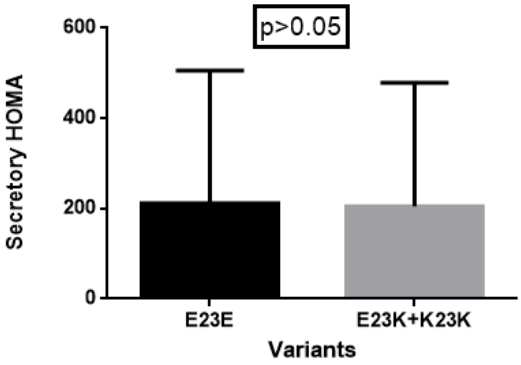


Figure 3.11: Secretory HOMA of Diabetic Study Subjects according to KCNJ11 Genotypes

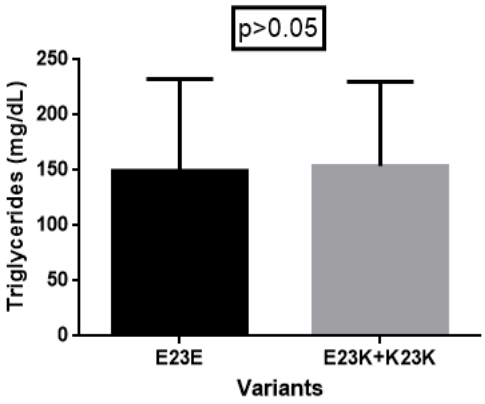


Figure 3.12: Triglycerides Level of Diabetic Study Subjects according to KCNJ11 Genotypes

The glucose **(Figure 3.10)** and triglyceride **(Figure 3.13)** levels were higher; HOMA B% **(Figure 3.11)** and Secretory HOMA **(Figure 3.12)** were lower in total mutant variants E23K+K23K in diabetic subjects compared to E23E variants, although the results were not statistically significant.

3.5.12 Univariate Logistic Regression Analysis for Risk Factors of Diabetes with KCNJ11 Genotype in Diabetic Subjects

To estimate the association of various clinical factors and diabetes, univariate logistic regression models were performed for gender, BMI, glucose, insulin, HOMA B%, HOMA S%, HOMA IR, triglycerides, total cholesterol, and HDL-Cholesterol with E23K and total mutant E23K+K23K variant of KCNJ11 genotype **(Table 3.19)**. The univariate logistic analyses indicated that BMI ($p<0.01$), glucose ($p<0.001$), triglycerides ($p<0.05$), total cholesterol ($p<0.01$), and HDL cholesterol ($p<0.001$) were significantly associated with diabetes. In subjects with E23K and total mutant E23K+K23K variants, Odd Ratio (OR) for diabetes were 1.768 (95% CI: 0.33 to 9.59, p=NS) and 0.175 (95% CI: 0.03 to 1.21, p= NS), respectively, which was showed that E23K variants had a 1.76-fold risk factor for diabetes.

Table 3.19: Univariate Logistic Regression Analysis for Risk Factors of Diabetes with KCNJ11 Genotype in Diabetic Subjects

Variables	B	SE	*p value*	OR	95% CI
Gender	0.356	0.292	*NS*	1.428	0.81-2.53
BMI (kg/m^2)	0.114	0.040	***<0.01***	1.121	1.04-1.21
Glucose (mM/L)	-3.229	0.536	***<0.001***	0.040	0.01-0.11
Insulin (µU/L)	-0.036	0.109	*NS*	0.964	0.78-1.19
HOMA B%	-0.020	0.008	*NS*	0.980	0.97-1.00
HOMA S%	-0.004	0.010	*NS*	0.996	0.98-1.02
HOMA IR	-0.090	0.984	*NS*	0.914	0.13-6.28
Triglycerides (mg/dL)	0.007	0.003	***<0.05***	1.007	1.00-1.01
T Cholesterol (mg/dL)	0.012	0.004	***<0.01***	1.012	1.00-1.02
HDL-C (mg/dL)	0.085	0.015	***<0.001***	1.089	1.06-1.12
KCNJ11 (E23K)	0.570	0.863	*NS*	1.768	0.33-9.59
KCNJ11 Genotype (E23K+K23K)	-1.745	0.985	*NS*	0.175	0.03-1.21

The reference category was E23E variants. *NS= Not Significant.*

3.5.13 Multiple Logistic Regression Analysis for Risk Factors of Diabetes with KCNJ11 Genotype in Diabetic Subjects

Multiple logistic regression analysis demonstrated that the genetic variant of KCNJ11 was non-significantly different between case and control after adjustment for potential confounders (**Table 3.20**). After adjusting for confounding factors, in subjects with E23K allele and total genetic variants, OR for diabetes was 0.749 (95% CI: 0.27 to 2.02) and 0.661 (95% CI: 0.21 to 1.99), respectively.

Table 3.20: Multiple Logistic Regression Analysis for Risk Factors of Diabetes with KCNJ11 Genotype in Diabetic Subjects

Variables	B	SE	*p value*	OR	95% CI
KCNJ11 (E23K)	-0.289	0.505	*NS*	0.749	0.278-2.02
KCNJ11 Genotype (E23K+ K23K)	-0.414	0.563	*NS*	0.661	0.219-1.99

Adjust with gender, BMI, glucose, insulin, HOMA B%, HOMA S%, HOMA IR, triglycerides, total cholesterol, and HDL cholesterol, respectively. *NS= Not Significant*

Study on
TCF7L2 Gene (rs12255372)

3.6 Determination of TCF7L2 (rs12255372) Gene Polymorphism

3.6.1 PCR Product for Detection of TCF7L2 Gene polymorphism

rs12255372, also known as IVS4G>T and c.483+9017G>T, is a well-studied SNP in the TCF7L2 gene on chromosome 10. A segment of the TCF7L2 gene was amplified, as shown in **Figure 3.14**. The amplified PCR products (99 bp) were evaluated by 2% agarose gel electrophoresis, and the size of the product was ascertained by comparing with 100 bp DNA ladder.

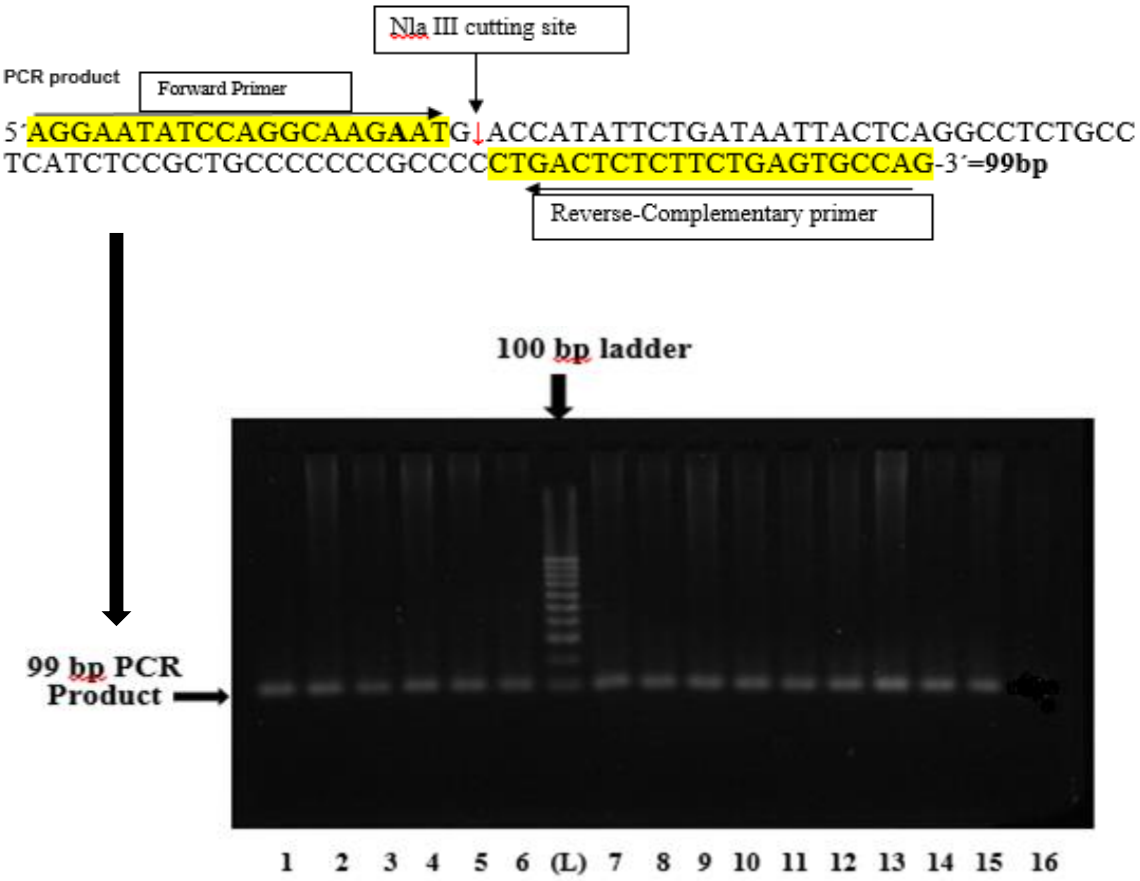


Figure 3.13: Representative PCR Products of TCF7L2 Gene in 2% Agarose Gel

Lane 1-6 and Lane 7-15 shows the presence of the PCR product of 99 bp. **Lane 16** shows the absence of PCR and **Lane L** 100 bp ladder.

3.6.2 Restriction Digestion of the PCR Product of TCF7L2 Gene

To determine the TCF7L2 genotype, amplified PCR products were digested with Hin1II (Nla III) for 12 hours. The final products were analyzed by electrophoresis in 3% agarose gel and visualized under UV light after EtBr staining. Mutation of the TCF7L2 gene causes G>T substitution, which is a candidate marker. The complete digestion (78 bp and 21 bp) indicated that both polymorphic alleles were wild type (GG). Incomplete digestion with Hin1II (Nla III) indicating heterozygote (Ht) mutant allele created 99 bp, 78 bp, and 21 bp fragment (GT) and absence of digestion with Hin1II (Nla III), indicating homozygote (Hz) mutant allele (TT) created fragments of only 99 bp band (**Figure 3.15**).

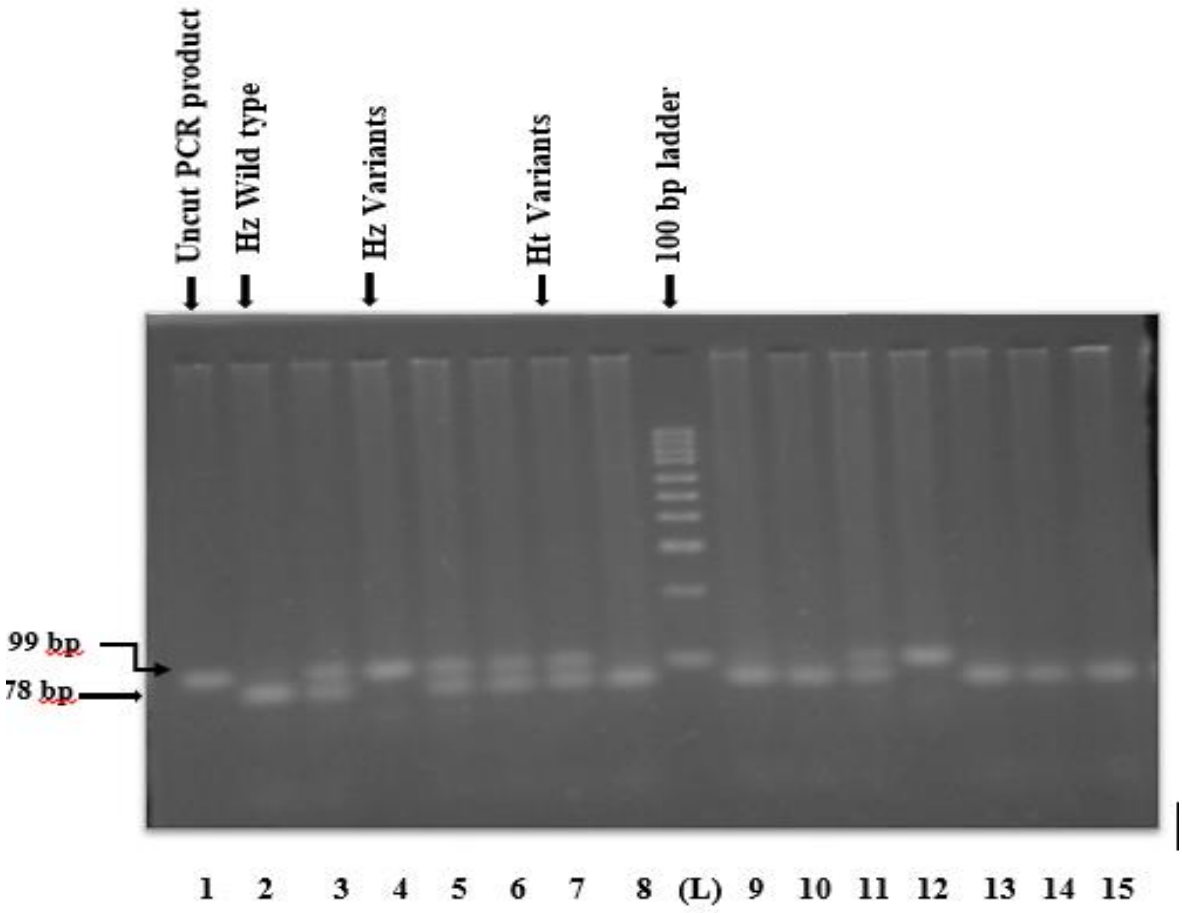


Figure 3.14: Representative Digested PCR Product of TCF7L2 Gene

Lane 1: undigested sample 99 bp, **Lane 2,8,9,10,13-15:** wild type 78 and 21 bp, **Lane 4, 12:** homozygous variants 99 bp, **Lane 3, 5,6,7,11:** heterozygous variants 99, 78 and 21 bp; The 21 bp fragment was not shown. **Lane L:** 100 bp DNA ladder.

3.6.3 Frequency Distribution of TCF7L2 Genotype and Risk of Diabetes

The distributions of the rs12255372 (G/T) polymorphism of the TCF7L2 gene in type 2 diabetic and control subjects and estimated risk was presented in **Table 3.21.** It was found that 57.9% of type 2 diabetic patients contained GG, 32.6% contained GT, and 9.4% contained TT genotypes. On the other hand, 66.6% of the control subjects had GG, 29.8% contained GT, and 3.7% contained TT genotypes, respectively. The difference in the genotype category GG vs. TT and GG vs. GT+TT among study subjects was statistically significant ($p<0.01$ and $p<0.05$, respectively). The T allele frequency was significantly ($p<0.05$) higher in diabetic subjects than control subjects when the frequency of the G allele was considered a reference group.

The risk for type 2 DM was also calculated in relation to the TCF7L2 gene. The frequency of the GG, GT, and TT genotypes in patients with type 2 diabetes were compared to that of the control subjects, and there was a significant relationship exist between GG vs. TT and GG vs. GT+TT [$p <0.01$, OR (95% CI): 2.94 (1.49-5.83) and $p<0.05$, OR (95% CI): 1.45 (1.06-1.97) respectively] when the GG genotype was considered as the reference group (**Table 3.21)**.

Table 3.21: Genotypic Distribution and Diabetic Risk Assessment of TCF7L2 Gene in the Study Subjects

Genotype	**Study Subjects**		**OR (95% CI)**	***p value***
	Diabetic (n=371) [n, %]	**Control (n=326) [n, %]**		
GG	215 (58.0)	217 (66.5)	**1 (Reference)**	**---**
GT	121 (32.6)	97 (29.8)	1.26 (0.91-1.75)	*NS*
TT	35 (9.4)	12 (3.7)	2.94 (1.49-5.83)	***<0.01***
GT+TT	156 (42.0)	109 (33.5)	1.45 (1.06-1.97)	***<0.05***
Allele	**[n, Frequency]**			
G allele	276 (0.742)	266 (0.814)		
T allele	96 (0.257)	61 (0.186)		***<0.05***

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. Odds ratios (OR) and 95% confidence interval (95% CI). $p<0.05$ was taken as the level of significance. TCF7L2 genotypes, wild type homozygote, GG; heterozygote variants, GT; homozygote variants, TT. *NS= Not Significant*.

3.6.4 Frequency Distribution of TCF7L2 Genotype and Risk of Diabetes according to gender

The genotypic distribution of TCF7L2, according to gender, is presented in **Table 3.22**. A significant difference was found for TT genotypes ($p<0.05$) in males, whereas no significant association was found for both GT and TT genotypes in female diabetic subjects when the GG genotype was considered as a reference group, respectively. Here TT variants may have a high risk for the predisposition of diabetes in males [$p <0.05$, OR (95% CI): 3.24 (1.31-7.98)] than female.

Table 3.22: Frequency distribution of TCF7L2 genotype and Risk of Diabetes according to gender

Gender	Genotype	Study Subjects		OR (95% CI)	*P value*
		Diabetic (n=371)	**Control (n=326)**		
		n=177 (n, %)	**n=172 (n, %)**		
Male	**GG**	98 (55.4)	111 (64.5)	**1 (Reference)**	**---**
	GT	59 (33.3)	54 (31.4)	1.24 (0.78-1.96)	*NS*
	TT	20 (11.3)	7 (4.1)	3.24 (1.31-7.98)	***<0.05***
		n=194 (n, %)	**n=154 (n, %)**		
Female	**GG**	117 (60.3)	106 (68.8)	**1 (Reference)**	**---**
	GT	62 (31.9)	43 (27.9)	1.23 (0.81-2.09)	*NS*
	TT	15 (7.7)	5 (3.2)	2.72 (0.96-7.74)	*NS*

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. Odds ratios (OR) and 95% confidence interval (95% CI). $p<0.05$ was taken as the level of significance. TCF7L2 genotypes, wild type homozygote, GG; heterozygote variants, GT; homozygote variants, TT. *NS= Not Significant*.

3.6.5 Frequency Distribution of TCF7L2 Genotype and Risk of Diabetes according to the Family History of Diabetes

The association between family history of diabetes and frequency of TCF7L2 genotype between diabetic and control subjects are presented in **Table 3.23.** The frequencies of TT variants of TCF7L2 genotypes was significantly higher in diabetic subjects with a positive family history of diabetes [$p<0.01$, OR (95% CI): 5.37 (1.58-18.28)], but no significant association was found for GT variants compared to GG variants in diabetic subjects with a positive family history of diabetes. Whereas, both GT and TT genotype showed no significant association in diabetic subjects without a family history of diabetes. The results suggested that the TT genotype of TCF7L2 may be a risk factor for the incidence of diabetes, which has a positive family history of diabetes.

Table 3.23: Frequency Distribution of TCF7L2 Genotype and Risk of Diabetes according to the Family History of Diabetes

Family History of diabetes	Genotype	Study Subjects		OR (95% CI)	*p value*
		Diabetic (n=371)	**Control (n=326)**		
		n=258 (n, %)	**n=132 (n, %)**		
YES	**GG**	146 (56.6)	94 (71.2)	**1 (Reference)**	**---**
	GT	87 (33.7)	35 (26.5)	1.60 (0.99-2.56)	*NS*
	TT	25 (9.7)	3 (2.3)	5.37 (1.58-18.28)	***<0.01***
		n=113 (n, %)	**n=194 (n, %)**		
NO	**GG**	69 (61.1)	123 (63.5)	**1 (Reference)**	**---**
	GT	34 (30.1)	62 (31.9)	0.98 (0.59-1.63)	*NS*
	TT	10 (8.8)	9 (4.6)	1.98 (0.77-5.11)	*NS*

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. Odds ratios (OR) and 95% confidence interval (95% CI). $p<0.05$ was taken as the level of significance. TCF7L2 genotypes, wild type homozygote, GG; heterozygote variants, GT; homozygote variants, TT. *NS= Not Significant*.

3.6.6 Influence of TCF7L2 Genotype on Risk of Diabetes with Age of Onset of Diabetes

Table 3.24 presents the risk of diabetes of TCF7L2 genotypes according to the onset of diabetes in a different age group. None of the variants were found significantly associated with diabetic risk with GT and TT genotypes, which may indicate that genotypic variants of TCF7L2 may not be associated with age. The incidence of diabetic can occur at any stage of life.

Table 3.24: Influence of TCF7L2 Genotype on Risk of Diabetes with Age of Onset of Diabetes

Age (years) of onset of diabetes	Genotype		OR (95% CI)
	GT+TT (n=156) (n, %)	GG (n=215) (n, %)	
<40 (n=111)	50 (32.0)	61 (28.4)	**1 (Reference)**
41-50 (n=150)	53 (34.0)	97 (45.1)	0.66 (0.40-1.10)
>50 (n=110)	53 (34.0)	57 (26.5)	1.13 (0.66-1.93)

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. Odds ratios (OR) and 95% confidence interval (95% CI). $p<0.05$ was taken as the level of significance. TCF7L2 genotypes, wild type homozygote, GG; heterozygote variants, GT; homozygote variants, TT.

3.6.7 TCF7L2 Genotype on Risk of Diabetes according to HbA1C Status

Association of TCF7L2 genotypes on the risk of diabetes, according to their HbA1C status, is presented in **Table 3.25.**

Table 3.25: TCF7L2 Genotype on Risk of Diabetes according to HbA1C Status

Genotype	HbA1C status in Diabetic Subjects		OR (95% CI)
	Severe (HbA_{1C}>8) (n=136) (%)	Mild/Moderate (HbA_{1C}<8) (n=235) (%)	
GG	74 (54.4)	141 (60.0)	**1 (Reference)**
GT	49 (36.0)	72 (30.6)	1.30 (0.82-2.05)
TT	13 (9.6)	22 (9.4)	1.13 (0.54-2.36)

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. Odds ratios (OR) and 95% confidence interval (95% CI). $p<0.05$ was taken as the level of significance. TCF7L2 genotypes, wild type homozygote, GG; heterozygote variants, GT; homozygote variants, TT.

The severity of diabetic was considered according to the status of the HbA1C level. From this table, it is clear that the severity of diabetes was not associated with TCF7L2 variants GT and TT, respectively.

3.6.8 TCF7L2 Genotype on Risk of Diabetes according to the Smoking Status

Table 3.26 presents the frequency of TC7L2 genotype in smoker and non-smoker of diabetic and control male subjects. The risk of development of diabetes was not associated with diabetic and non-diabetic smoker group. Whereas, the frequency of the TT genotype was significantly higher [OR (95% CI) = 4.145 (1.269-13.54); $p<0.05$] in non-smoker diabetic patients compared to control. It suggested no possible association of smoking status with diabetic development in GT and TT genotypes.

Table 3.26: TCF7L2 Genotype on Risk of Diabetes according to the Smoking Status

Smoking Status	**Genotype**	**Study Subjects**		**OR (95% CI)**	***p value***
		Diabetic (n=177)	**Control (n=172)**		
		n=72 (%)	**n=55 (%)**		
Smoker	**GG**	43 (60)	35 (64)	**1 (Reference)**	**---**
	GT	21 (29)	17 (31)	1.005 (0.461-2.193)	*NS*
	TT	8 (11)	3 (5)	2.171 (0.535-8.804)	*NS*
		n=105 (%)	**n=117 (%)**		
Non Smoker	**GG**	55 (52)	76 (65)	**1 (Reference)**	**---**
	GT	38 (36)	37 (32)	1.419 (0.802-2.511)	*NS*
	TT	12 (12)	4 (3)	4.145 (1.269-13.54)	**<0.05**

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. Odds ratios (OR) and 95% confidence interval (95% CI). $p<0.05$ was taken as the level of significance. TCF7L2 genotypes, wild type homozygote, GG; heterozygote variants, GT; homozygote variants, TT. *NS= Not Significant*.

3.6.9 TCF7L2 Genotype on Risk of Diabetes according to the area of Residence

The association of area of residence and frequency of TCF7L2 genotype between diabetic subjects and controls is presented in **Table 3.27.** The frequency of GT and TT variants of TCF7L genotypes were not associated with diabetic subjects living in the rural area. GT and TT variants showed a significant association with diabetic subjects living in semi-urban [p<0.05, OR (95% CI); 2.66 (1.17-6.02)] and urban [p<0.05, OR (95% CI); 2.71 (1.23-5.94)] area when GG variant was considered as reference group. The result indicated that GT and TT genotypes of TCF7L2 might increase the risk factor for the predisposition of diabetes in semi-urban and urban populations, respectively, that may occur due to urbanization.

Table 3.27: TCF7L2 Genotype on Risk of Diabetes according to the Area of Residence

Area of Residence	Genotype	Study Subjects		OR (95% CI)	*p value*
		Diabetic (n=371)	**Control (n=326)**		
		n=59 (%)	**n=7 (%)**		
Rural	**GG**	33 (55.93)	5 (71.43)	**1 (Reference)**	**---**
	GT	16 (27.11)	1 (14.29)	2.42 (0.26-22.52)	*NS*
	TT	10 (16.94)	1 (14.29)	1.51 (0.15-14.54)	*NS*
		n=85 (%)	**n=42 (%)**		
Semi Urban	**GG**	41 (48.24)	30 (71.43)	**1 (Reference)**	**---**
	GT	40 (47.06)	11 (26.19)	2.66 (1.17-6.02)	***<0.05***
	TT	4 (4.71)	1 (2.38)	2.93 (0.3127.54)	*NS*
		n=227 (%)	**n=277 (%)**		
Urban	**GG**	141 (62.11)	182 (65.70)	**1 (Reference)**	**---**
	GT	65 (28.63)	85 (30.69)	0.99 (0.66-1.46)	*NS*
	TT	21 (9.25)	10 (3.611)	2.71 (1.23-5.94)	***<0.05***

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. Odds ratios (OR) and 95% confidence interval (95% CI). p<0.05 was taken as the level of significance. TCF7L2 genotypes, wild type homozygote, GG; heterozygote variants, GT; homozygote variants, TT. *NS= Not Significant*.

3.6.10 Distributions of Glycemic and Insulinemic Status according to TCF7L2 Genotype in the Study Subjects

Distribution of glycemic and insulinemic status according to TCF7L2 genotypes in the study subjects are presented in **Table 3.28**. HbA1c% (8.0±1.5) and GINR (0.50±0.34) were higher in the TT genotype in comparison to GG (7.7±1.7% and 0.40±0.30 respectively) and GT (7.9±1.7% and 0.42±0.26 respectively) genotype in diabetic subjects, but not statistically significant.

Serum fasting insulin level, HOMA B%, secretory HOMA, and HOMA IR were decreased in TT homozygous variants than GG and GT genotype in diabetic subjects, but values were not statistically significant. A significant increase of QUICKI was found in TT variants in the diabetic group ($p<0.05$) compared with GG and GT variants.

3.6.11 Distribution of Lipidemic, ALT, and Creatinine Status according to TCF7L2 Genotype in the Study Subjects

Association of lipid levels, according to TCF7L2 genotypes in study subjects are shown in **Table 3.29**. Serum triglycerides level was higher in TT genotype (172±128 mg/dL) in diabetic subjects compared to GG (151±74 mg/dL) and GT (146±71 mg/dL) respectively, but not statistically significant. Whereas, total cholesterol, LDL-cholesterol, HDL-cholesterol levels, and total cholesterol and HDL ratio did not show any expected variation among GG, GT, and TT genotype in diabetic and control subjects.

There were no significant variations found in serum ALT and creatinine levels with GG, GT, and TT genotype in TCF7L2 genes in diabetic and control subjects.

Table 3.28: Distributions of Glycemic and Insulinemic Status according to TCF7L2 Genotype in the Study Subjects

Variables	*Study Subjects*							
	Diabetic (n=371)			*p value*	*Control (n=326)*			*p value*
	GG *(n=215)*	**GT *(n=121)***	***TT (n=35)***		**GG *(n=217)***	**GT *(n=97)***	***TT (n=12)***	
Glucose (mM/L)	7.5±2.7	7.6±2.5	7.3±2.0	*NS*	4.9±0.6	5.1±0.6	4.9±0.5	*NS*
HbA1c (%)	7.7±1.7	7.9±1.7	8.0±1.5	*NS*	5.1±0.5	5.1±0.6	5.0±0.6	*NS*
Insulin (µU/L)	24.8±14.2	23.4±14.9	19.3±11.5	*NS*	14.3±5.9	14.7±5.6	14.1±5.4	*NS*
GINR	0.40±0.30	0.42±0.26	0.50±0.34	*NS*	0.45±0.32	0.44±0.33	0.40±0.15	*NS*
HOMA B%	123.9±84.7	112.9±76.9	95.8±47.5	*NS*	150.4±58.1	145.7±51.7	151.1±53.9	*NS*
HOMA S%	40.4±26.4	40.7±19.3	50.8±30.4	*NS*	70.6±47.7	66.9±46.7	63.0±21.5	*NS*
HOMA IR	3.3±1.8	3.2±1.8	2.7±1.5	*NS*	1.8±0.7	1.8±0.7	1.8±0.7	*NS*
QUICKI	0.29±0.03	0.29±0.02	0.30±0.03	***<0.05***	0.33±0.03	0.33±0.03	0.33±0.02	*NS*
Secretory HOMA	233.1±343.1	182.02±173.4	152.0±143.6	*NS*	271.4±194.0	245.0±151.6	260.7±145.5	*NS*

Results are presented as mean ± standard deviation (SD). Groups of data were compared using a one-way ANOVA test. Differences were considered significant at $p<0.05$. GINR: Glucose insulin Ratio; HOMA B%= Beta Cell Function; HOMA S%= Beta Cell Sensitivity; HOMA-IR: Homeostasis Model of Assessment - Insulin Resistance; QUICKI= Quantitative Insulin Sensitivity Check Index; Secretory HOMA: Secretory Homeostasis Model of Assessment.TCF7L2 genotypes, wild type homozygote, GG; heterozygote variants, GT; homozygote variants, TT. *NS= Not Significant*.

Table 3.29: Distributions of Lipidemic. ALT and Creatinine Status according to TCF7L2 Genotype in the Study Subjects

Variables	*Study Subjects*							
	Diabetic (n=371)			*p value*	*Control (n=326)*			*p value*
	GG *(n=215)*	**GT *(n=121)***	***TT (n=35)***		**GG *(n=217)***	**GT *(n=97)***	***TT (n=12)***	
Triglycerides (mg/dL)	151±74	146±71	172±128	*NS*	133±47	133±46	130±60	*NS*
Total cholesterol (mg/dL)	169±52	164±51	160±66	*NS*	170±32	174±33	169±38	*NS*
HDL-Cholesterol (mg/dl)	41±11	39±10	42±9	*NS*	52±14	50±14	48±14	*NS*
LDL-cholesterol (mg/dL)	110±46	105±42	101±63	*NS*	94±34	96±32	94±28	*NS*
TC: HDL-C	4.7±3.7	4.4±1.9	4.0±2.0	*NS*	3.6±1.0	3.8±1.3	3.9±1.5	*NS*
ALT (U/L)	29±18	29±13	28±14	*NS*	29±8	30±11	32±5	*NS*
Creatinine (mg/dL)	0.92±0.11	0.93±0.12	0.93±0.13	*NS*	0.94±0.12	0.93±0.11	0.94±0.12	*NS*

Results are presented as mean ± standard deviation (SD). Groups of data were compared using a one-way ANOVA test. Differences were considered significant at $p<0.05$. ALT= Analine Aminotransferase, TC:HDL-C: Total cholesterol: HDL-Cholesterol ratio. TCF7L2 genotypes, Wild type homozygote, GG; heterozygote variants, GT; homozygote variants, TT. *NS= Not Significant*.

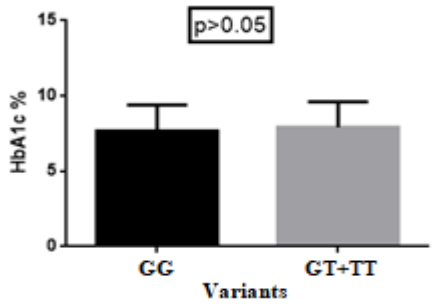


Figure 3.15: HbA1C% of Diabetic Study Subjects according to TCF7L2 Genotypes

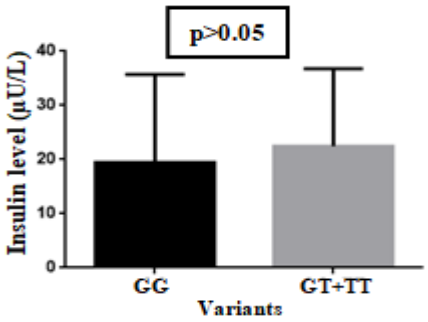


Figure 3.16: Insulin Level of Diabetic Study Subjects according to TCF7L2 Genotypes

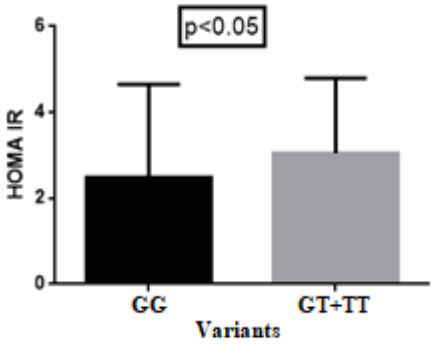


Figure 3.17: HOMA IR of Diabetic Study Subjects according to TCF7L2 Genotypes

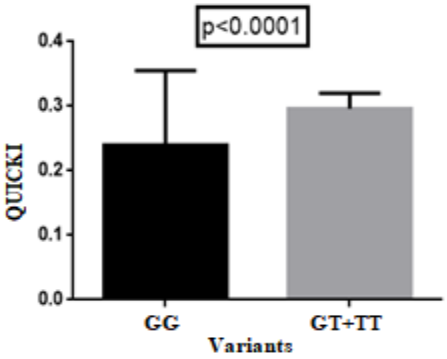


Figure 3.18: QUICKI of Diabetic Study Subjects according to TCF7L2 Genotypes

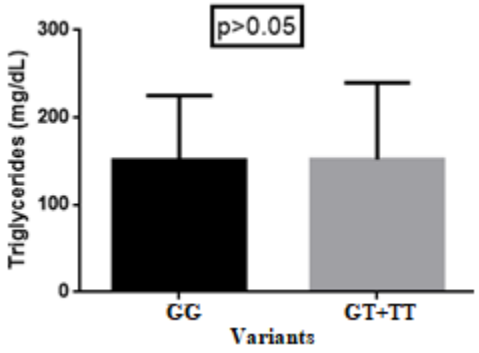


Figure 3.19: Triglycerides Level of Diabetic Study Subjects according to TCF7L2 Genotypes

Associations were also found for HbA1C, insulin, HOMA IR, QUICKI, and triglycerides with total variant GT+TT when considered the GG variant as a reference. As shown in **Figure 3.15, Figure 3.16,** and **Figure 3.19** that HbA1C, insulin, and triglyceride values

were higher in variant GT+TT in comparison to GG, but the increased values were not statistically significant. In contrast, the value of HOMA IR and QUICKI were significantly higher ($p<0.05$ and $p<0.001$ respectively) in GT+TT variants than the GG variant (**Figure 3.17** and **Figure 3.18**).

3.6.12 Univariate Logistic Regression Analysis for Risk Factors of Diabetes with TCF7L2 Genotype in Diabetic Subjects

To estimate the association of various clinical factors and diabetes, univariate logistic regression models were performed for gender, BMI, SBP, DBP, family history of diabetes, glucose, insulin, HOMA B%, HOMA S%, HOMA IR, triglycerides, total cholesterol, and HDL-Cholesterol parameters; and GT and total GT+TT variants of TCF7L2 gene (**Table 3.30**).

Table 3.30: Univariate Logistic Regression Analysis for Risk Factors of Diabetes with TCF7L2 Genotype in Diabetic Subjects

Variables	B	SE	*p value*	OR	95% CI
Gender	0.547	0.325	*NS*	1.727	0.91-3.26
BMI (Kg/m^2)	0.109	0.042	***<0.05***	1.115	1.03-1.21
SBP (mmHg)	-0.026	0.016	*NS*	0.975	0.94-1.01
DBP (mmHg)	-0.034	0.030	*NS*	0.966	0.91-1.03
Family history	-1.107	0.311	***<0.001***	0.330	0.18-0.61
Glucose (mM/L)	-3.098	0.559	***<0.001***	0.045	0.02-0.14
Insulin (µU/L)	-0.105	0.137	*NS*	0.901	0.69-1.18
HOMA B%	-0.020	0.008	***<0.05***	0.980	0.97-1.00
HOMA S%	-0.006	0.009	*NS*	0.994	0.98-1.01
HOMA IR	0.467	1.167	*NS*	1.595	0.16-15.71
Triglycerides (mg/dL)	0.004	0.003	*NS*	1.004	0.99-1.01
Cholesterol (mg/dL)	0.014	0.004	***<0.01***	1.014	1.01-1.02
HDL-C (mg/dL)	0.072	0.016	***<0.001***	1.075	1.04-1.10
TCF7L2 (G>T)	-1.557	0.661	***<0.05***	0.211	0.06-0.77
TCF7L2 Genotype (GT+TT)	1.578	0.841	*NS*	4.846	0.93-25.20

The reference category was GG variants group. *NS=Not Significant*.

The univariate logistic analyses indicated that BMI ($p<0.05$), family history of diabetes ($p<0.001$), glucose level ($p<0.001$), HOMA B% ($p<0.05$), total cholesterol ($p<0.01$), and HDL-Cholesterol ($p<0.001$) were significantly associated with diabetes. In diabetic subjects, the GT variants OR was 0.211 (95% CI: 0.06 to 0.77, $p<0.05$) and the GT+TT variants OR was 4.846 (95% CI: 0.93 to 25.20, p= NS) respectively. It may be suggested that GT variants have a high risk for diabetes.

3.6.13 Multiple Logistic Regression Analysis for Risk Factors of Diabetes with TCF7L2 Genotype in Diabetic Subjects

Multiple logistic regression analysis demonstrated that the genetic variant of TCF7L2 remained significantly different between diabetes and control subjects after adjustment for the potential confounders ($p<0.05$ for allele analysis) **(Table 3.31)**. After adjust for confounding factors, in diabetic subjects with TCL7L2 (GT) and genetic variants (GT+TT), the OR for diabetes were 0.428 (95% CI: 0.21 to 0.87, $p<0.05$) and 1.857 (95% CI: 0.78 to 4.42, p=NS) respectively. It also showed that GT variants have a higher risk for diabetes when adjusting with potential confounders.

Table 3.31: Multiple Logistic Regression Analysis for Risk Factors of Diabetes with TCF7L2 Genotype in Diabetic Subjects

Variables	B	SE	p value	OR	95% CI
TCF7L2 (G>T)	-0.849	0.361	***<0.05***	0.428	0.21-0.87
TCF7L2 Genotype (GT+TT)	0.619	0.442	*NS*	1.857	0.78-4.42

Adjust with gender, BMI, SBP, DBP, family history of diabetes, glucose, insulin, HOMA B%, HOMA S%, HOMA IR, triglycerides, total cholesterol, and HDL cholesterol respectively. *NS=Not Significant.*

Study On
SLC22A1 Gene (rs628031)

3.7 Determination of SLC22A1 (rs628031) Gene Polymorphism

3.7.1 PCR Product for Detection of SLC22A1 Gene Polymorphism

By using specific primers, PCR was done where a segment of the SLC22A1 gene was amplified, as shown in **Figure 3.22**. The amplified PCR products (422 bp) were evaluated by 2% agarose gel electrophoresis, and the size of the product was ascertained by comparing it with 100 bp DNA ladder.

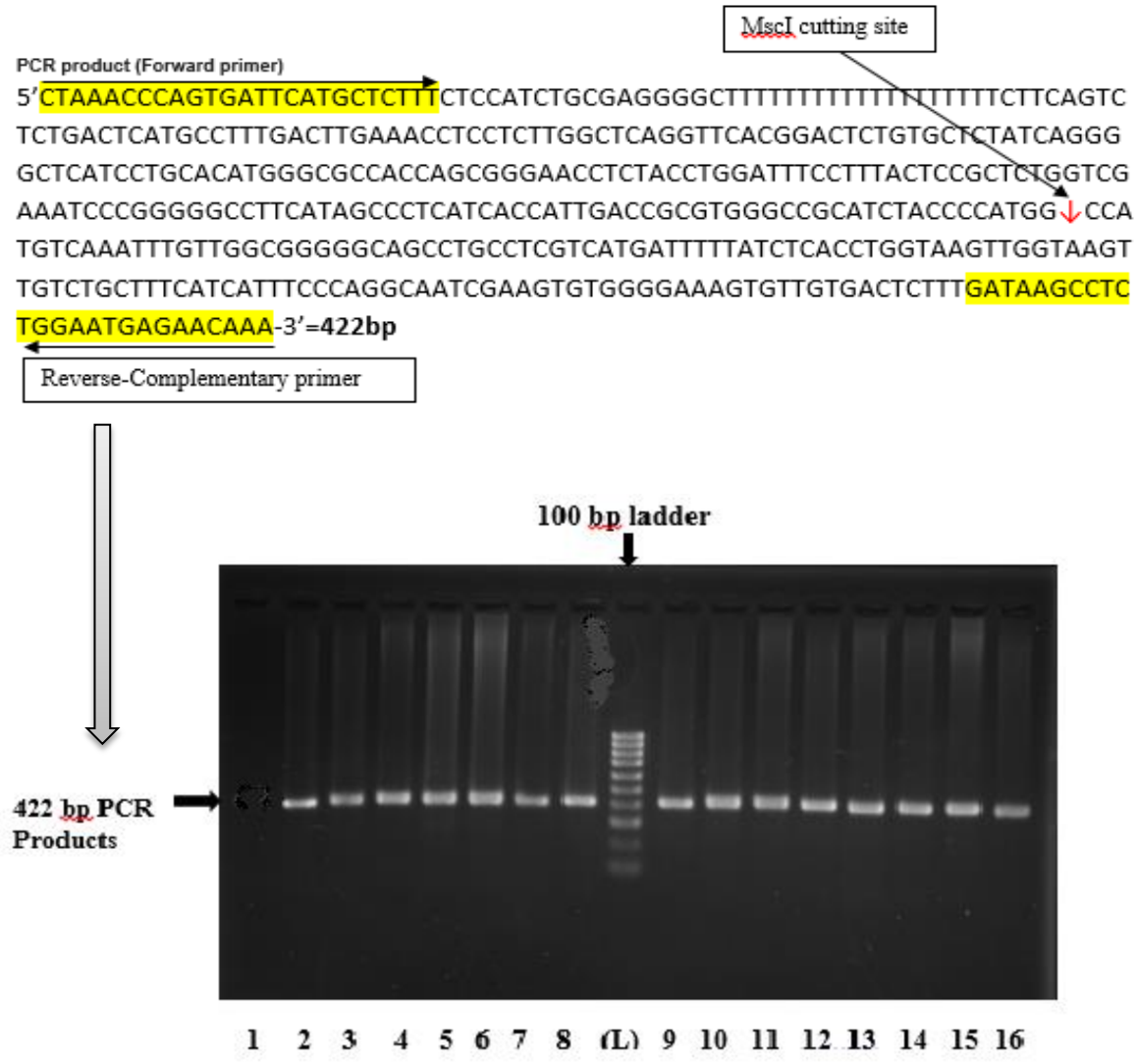


Figure 3.20: Representative PCR Products of SLC22A1 Gene in 2% Agarose Gel

Lane 1: Absence of PCR and **Lane 2-8 and Lane 9-16:** Presence of the PCR product of 422 bp.

Lane L: 100 bp ladder.

3.7.2 Restriction Digestion of the PCR Product of SLC22A1 Gene

To determine the SLC22A1 genotype, amplified PCR products were digested with MlsI (MscI) for 12 hours. The final products were analyzed by electrophoresis in 2% agarose gel and visualized under UV light after EtBr staining. Mutation of the SLC22A1 gene causes G>A substitution, which is a candidate marker. The complete digestion (68 bp and 154 bp) indicated that both polymorphic alleles were wild type (GG). Incomplete digestion with MlsI (MscI) indicating heterozygote (Ht) variants allele (GA) created 422 pb, 268 pb and 154 bp fragment and absence of digestion with MlsI (MscI), indicating homozygote (Hz) variants allele (AA) created fragments of only 422 bp band (**Figure 3.23**).

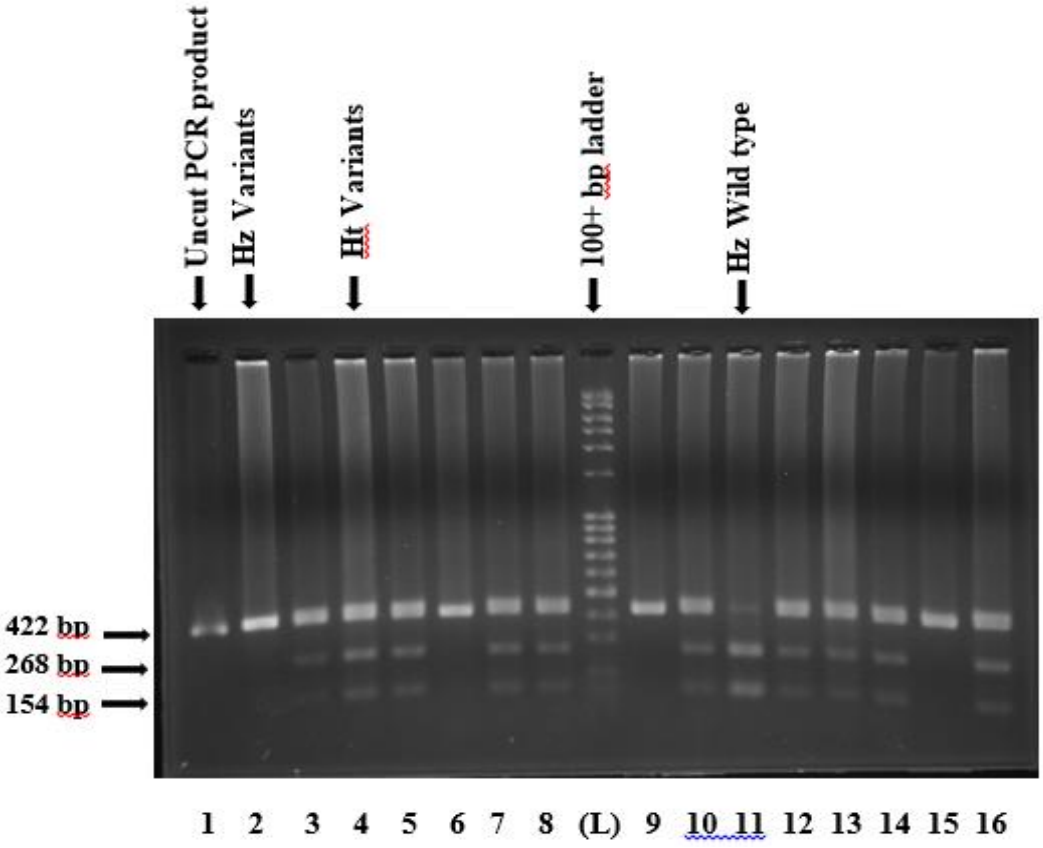


Figure 3.21: Representative Digested PCR Product of SLC22A1 Gene

Lane 1: undigested sample 422 bp, **Lane 11**: wild type 268 and 154 bp, **Lane 2,6,9,15**: homozygous variants 422 bp, **Lane 3-5,7-8,10,12-14,16:** heterozygous variants 422, 268 and 154 bp and **Lane L:** 100+ bp DNA ladder.

3.7.3 Frequency Distribution of SLC22A1 Genotype and Risk of Diabetes

The distributions of SLC22A1 genotype in type 2 diabetic and control subjects and estimated risk are presented in **Table 3.32**. It was found that 7.3% of type 2 diabetic patients contained GG, 58.0% contained GA, and 34.8% had AA genotypes. Whereas, 27.3% of the control subjects contained GG, 42.0% contained GA, and 30.7% contained AA genotypes, respectively. GA, AA, and dominant model GA+AA variants were significantly higher ($p<0.001$ for all) in diabetic study subjects than control, when the GG variant was considered a reference group. SLC22A1 variant allele frequency was 0.483 and 0.426 for wild and mutant variants respectively in control, and 0.363 and 0.247 for diabetic subjects, respectively. "A" allele frequency was significantly ($p<0.01$) higher in diabetic subjects than control subjects. The risk for T2DM was also calculated in relation to the SLC22A1 gene. The frequency of the GG, GA, and AA genotypes in patients with type 2 diabetes was compared to that of the control subjects. There was a significant relationship between GG vs. GA ($p <0.001$), GG vs. AA ($p <0.001$), and GG vs. GA+AA ($p <0.001$) when the GG genotype was considered as reference group respectively (**Table 3.32**).

Table 3.32: Genotypic Distribution and Diabetic Risk Assessment of SLC22A1 Gene in the Study Subjects

	Study Subjects			
Genotype	**Diabetic (n=371) (n, %)**	**Control (n=326) (n, %)**	**OR (95% CI)**	***p value***
GG	27 (7.3)	89 (27.3)	**1 (Reference)**	**---**
GA	215 (58.0)	137 (42.0)	5.17 (3.20-8.37)	***<0.001***
AA	129 (34.8)	100 (30.7)	4.25 (2.57-7.04)	***<0.001***
GA+AA	344 (92.72)	237 (72.70)	4.78 (3.02-7.59)	***<0.001***
Allele	***(*n, Frequency)**			
G allele	135 (0.363)	158 (0.483)		**---**
A allele	236 (0.247)	168 (0.426)		***<0.01***

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. $p<0.05$ was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). SLC22A1 codon GG-wild-type homozygote; GA, heterozygote variant; AA, homozygote variant.

3.7.4 Frequency Distribution of SLC22A1 Genotypic and Risk of Diabetes according to gender

The genotypic distribution of SLC22A1, according to gender, is represented in **Table 3.33**. A significant association of GA and AA variants was found in both diabetic males [GG vs. GA, OR (95% CI)**,** 3.61 (1.76-7.40), p<0.001 and GG vs. AA, OR (95% CI)**,** 3.04 (1.43-6.48), p<0.01] and female [GG vs. GA, OR (95% CI)**,** 7.47 (3.87-14.44), p<0.001 and GG vs. AA, OR (95% CI)**,** 5.66 (2.86-11.18), p<0.001] subjects, when GG genotype was considered as reference group respectively. This may be described that both GA and AA variants of the SLC22A1 gene have a high risk for the predisposition of diabetes in males and females, respectively.

Table 3.33: Frequency Distribution of SLC22A1 Genotype in the Study Subjects according to gender

Gender	Genotype	Study Subjects		OR (95% CI)	*p value*
		Diabetic (n=371)	**Control (n=326)**		
		n=177 (n, %)	**n=172 (n, %)**		
Male	**GG**	12 (6.8)	34 (19.8)	**1 (Reference)**	**---**
	GA	107 (60.4)	84 (48.8)	3.61 (1.76-7.40)	***<0.001***
	AA	58 (32.8)	54 (31.4)	3.04 (1.43-6.48)	***<0.01***
		n=194 (n, %)	**n=154 (n, %)**		
Female	**GG**	15 (7.7)	55 (35.7)	**1 (Reference)**	**---**
	GA	108 (55.7)	53 (34.4)	7.47 (3.87-14.44)	***<0.001***
	AA	71 (36.6)	46 (29.9%)	5.66 (2.86-11.18)	***<0.001***

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. p<0.05 was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). SLC22A1 codon GG- wild-type homozygote; GA, heterozygote variant; AA, homozygote variant.

3.7.5 Frequency Distribution of SLC22A1 Genotype and Risk of Diabetes according to the Family History of Diabetes

The association between family history of diabetes and frequency of SLC22A1 genotype between diabetic subjects and controls are presented in **Table 3.34.** As shown in **Table 3.34,** the frequencies of GA and AA variants of SLC22A1 genotypes were significantly higher in diabetic subjects with a positive family history of diabetes [GG vs. GA; OR (95% CI): 4.54 (2.46-8.35), p<0.001 and GG vs. AA; OR (95% CI): 3.67 (1.91-7.02), p<0.001] and as well as in diabetic subjects without a family history of diabetes [GG vs. GA; OR (95% CI): 8.22 (3.10-21.78), p<0.001 and GG vs. AA; OR (95% CI); 7.50 (2.77-20.32), p<0.001] when GG variant was considered as reference group respectively. These results indicated that GA and AA variants were highly associated with/without a positive family history of diabetes. It may have a higher risk of predisposing to diabetes.

Table 3.34: Frequency Distribution of SLC22A1 Genotype and Risk of Diabetes according to the Family History of Diabetes

Family History of Diabetes	Genotype	Study Subjects		OR (95% CI)	*p value*
		Diabetic (n=371)	**Control (n=326)**		
		n=258 **(n, %)**	***n =132*** **(n, %)**		
YES	**GG**	22 (8.5)	37 (28.0)	**1 (Reference)**	**---**
	GA	151 (58.5)	56 (42.4)	4.54 (2.46-8.35)	***<0.001***
	AA	85 (32.9)	39 (29.5)	3.67 (1.91-7.02)	***<0.001***
		n=133 **(n, %)**	***n=194*** **(n, %)**		
NO	**GG**	5 (3.8)	52 (26.8)	**1 (Reference)**	**---**
	GA	64 (48.1)	81 (41.5)	8.22 (3.10-21.78)	***<0.001***
	AA	44 (33.1)	61 (31.4)	7.50 (2.77-20.32)	***<0.001***

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. p<0.05 was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). SLC22A1 codon GG- wild-type homozygote; GA, heterozygote variant; AA, homozygote variant.

3.7.6 Influence of SLC22A1 Genotype on Risk of Diabetes with Age of Onset of Diabetes

Table 3.35 presented SLC22A1 genotypes on the risk of diabetes according to the onset of diabetes in different age groups. None of the variants showed significant association with diabetic risk in diabetic subjects with GA and AA genotypes, which may be explained that genotypic variants of SLC22A1 may not be associated with the onset of diabetes.

Table 3.35: Influence of SLC22A1 Genotype on Risk of Diabetes with Age of Onset of Diabetes

Age (years) for onset of Diabetes	Genotype		OR (95% CI)
	GA +AA (n= 344) (n, %)	GG (n=27) (n, %)	
<40 (n=111)	103 (29.9)	8 (29.6)	**1 (Reference)**
41-50 (n=150)	136 (39.5)	14 (51.9)	0.75 (0.31-1.87)
>50 (n=110)	105 (30.6)	5 (18.5)	1.63 (0.52-5.15)

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. $p<0.05$ was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). SLC22A1 codon GG- wild-type homozygote; GA, heterozygote variant; AA, homozygote variant.

3.7.7 SLC22A1 Genotype on Risk of Diabetes according to HbA1C Status

Association of SLC22A1 genotypes on the risk of diabetes according to their HbA1C status is presented in **Table 3.36**.

Table 3.36: SLC22A1 Genotype on Risk of Diabetes according to HbA1C Status

Genotype	HbA_{1C} Status of Diabetic Subjects		
	Severe (n=136) (n, %)	Mild/Moderate (n=235) (n, %)	OR (95% CI)
GG	12 (8.8)	15 (6.4)	**1 (Reference)**
GA	80 (58.8)	135 (57.4)	1.35 (0.60-3.03)
AA	44 (32.4)	85 (36.2)	1.55 (0.67-3.59)

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. $p<0.05$ was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). SLC22A1 codon GG- wild-type homozygote; GA, heterozygote variant; AA, homozygote variant.

No association was found in severe and mild/moderate diabetic subjects with genotypic variants. As shown in **Table 3.36**, it may be suggested that the severity of diabetes is not associated with GA and AA variants of the SLC22A1 genotype, respectively.

3.7.8 SLC22A1 Genotype on Risk of Diabetes according to the Smoking Status

Table 3.37 presents the frequency of SLC22A1 genotypes in smoker and non-smoker category of diabetic and non-diabetic male subjects. No significant association was found in this study on smoker diabetic and control subjects. Whereas, the frequency of GA and AA genotypes was unexpectedly higher in non-smoker diabetic patients compared to control, respectively.

Table 3.37: SLC22A1 Genotype on Risk of Diabetes according to the Smoking Status

Smoking Status	Genotype	Study Subjects		OR (95% CI)	*p value*
		Diabetic (n=177)	**Control (n=172)**		
		n=72 (n, %)	**n=55 (n, %)**		
Smoker	**GG**	6 (8.3)	7 (12.7)	**1 (Reference)**	**---**
	GA	47 (65.3)	30 (54.6)	1.828 (0.560-5.966)	*NS*
	AA	19 (26.37)	18 (32.7)	1.231 (0.347-4.372)	*NS*
		n=105 (n, %)	**n=117 (n, %)**		
Non-Smoker	**GG**	6 (5.7)	27 (23)	**1 (Reference)**	**---**
	GA	60 (57.1)	54 (46)	5.000 (1.918-13.03)	*NS*
	AA	39 (37.2)	36 (31)	4.875 (1.804-13.17)	***<0.01***

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. $p<0.05$ was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). SLC22A1 codon GG- wild-type homozygote; GA, heterozygote variant; AA, homozygote variant. *NS=Not Significant*.

3.7.9 SLC22A1 Genotype on Risk of Diabetes according to the area of Residence

According to the study subjects' area of residence, the association of the frequency of SLC22A1 genotype between diabetic and control subjects is presented in **Table 3.38.**

The frequency of GA and AA variants of SLC22A1 genotypes were not associated with diabetic and control subjects living in the rural area. Whereas, GA and AA variants showed a higher significant association in both semi-urban [GG vs GA, OR (95% CI); 6.47 (1.96-21.31), p<0.01 and GG vs AA, OR (95%CI): 6.47 (1.96-21.31), p<0.05] and urban [GG vs GA, OR (95% CI); 4.44 (2.54-7.79), p<0.001; and GG vs AA, OR (95%CI): 3.71 (2.06-6.69), p<0.001] diabetic subjects than control, where GG genotype was considered as a reference respectively. The result indicated that GA and AA genotypes of SLC22A1 might increase the risk factor for semi-urban and urban diabetic subjects due to urbanization factors like environmental pollution, food habit or food chemical or toxicity, etc.

Table 3.38: SLC22A1 Genotype on Risk of Diabetes according to the Area of Residence

Area of Residence	Genotype	Study Subjects		OR (95% CI)	*p value*
		Diabetic (n=371)	Control (n=326)		
Rural		***n=59* (n, %)**	***n=7* (n, %)**		
	GG	3 (5.08)	2 (28.57)	**1 (Reference)**	**---**
	GA	35 (59.32)	3 (42.86)	7.77 (0.91-66.33)	*NS*
	AA	21 (35.59)	2 (28.57)	7.00 (0.69-70.09)	*NS*
Semi Urban		***n=85* (n, %)**	***n=42* (n, %)**		
	GG	5 (5.88)	11 (26.19)	**1 (Reference)**	**---**
	GA	50 (58.82)	17 (40.48)	6.47 (1.96-21.31)	***<0.01***
	AA	30 (35.29)	14 (33.33)	4.71 (1.37-16.18)	***<0.05***
Urban		***n=227* (n, %)**	***n=277* (n, %)**		
	GG	19 (8.37)	76 (27.43)	**1 (Reference)**	**---**
	GA	130 (57.27)	117 (42.24)	4.44 (2.54-7.79)	***<0.001***
	AA	78 (34.36)	84 (30.32)	3.71 (2.06-6.69)	***<0.001***

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association between diabetic and control subjects for the genotype frequencies. p<0.05 was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). SLC22A1 codon GG-wild-type homozygote; GA, heterozygote variant; AA, homozygote variant.

3.7.10 Distributions of Glycemic and Insulinemic Status according to SLC22A1 Genotypes in the Study Subjects

Distribution of glycemic and insulinemic status according to SLC22A1 genotypes in study subjects is presented in **Table 3.39**. HOMA IR was decreased in AA genotype (3.2±1.9) than GG (3.0±1.7) and GA (3.0±1.8) genotypes in diabetic subjects, respectively. HbA1C%, GINR, and other parameters showed no variation according to genotypes between the two groups.

3.7.11 Distributions of Lipidemic Status according to SLC22A1 Genotype in the Study Subjects

Association of lipid profiles, according to SLC22A1 genotypes in study groups, is presented in **Table 3.40**. The serum triglyceride level did not change based on genotypes. Total cholesterol and LDL-Cholesterol levels were comparatively higher, and HDL-Cholesterol was lower in GG genotype in diabetic subjects than GA and AA genotype, respectively. The results suggested that no association exists in lipidemic status with SLC22A1 genotypes.

There were no significant variations found in serum ALT and creatinine levels with GG, GA, and AA genotype in SLC22A1 genes in diabetic and control subjects.

Table 3.39: Distributions of Glycemic and Insulinemic Status according to SLC22A1 Genotype in the Study Subjects

Variables	*Study Subjects*							
	Diabetes (n=371)				*Control (n=326)*			
	GG *(n=27)*	**GA *(n=215)***	***AA (n=129)***	***p value***	**GG *(n=89)***	**GA *(n=137)***	***AA (n=100)***	***p value***
Glucose (mM/L)	7.4±2.6	7.6±2.6	7.3±2.5	*NS*	4.8±0.6	5.0±0.5	4.9±0.7	*NS*
HbA1c (%)	7.9±2.1	7.8±1.8	7.7±1.6	*NS*	5.1±0.5	5.1±0.5	5.1±0.5	*NS*
Insulin (µU/L)	23.1±13.3	23.7±13.4	24.2±15.7	*NS*	13.8±6.3	14.7±5.5	14.6±7.8	*NS*
GINR	0.44±0.30	0.41±0.28	0.41±0.31	*NS*	0.48±0.39	0.42±0.26	0.44±0.32	*NS*
HOMA B%	111.3±70.9	112.1±67.9	128.4±97.1	*NS*	151.0±57.9	144.4±51.1	153.5±60.5	*NS*
HOMA S%	47.4±34.2	40.9±25.5	41.1±21.3	*NS*	76.4±55.1	64.7±38.1	69.1±48.9	*NS*
HOMA IR	3.0±1.7	3.2±1.8	3.2±1.9	*NS*	1.8±0.8	1.9±0.7	1.9±0.7	*NS*
QUICKI	0.30±0.03	0.29±0.03	0.30±0.02	*NS*	0.33±0.03	0.33±0.02	0.33±0.03	*NS*
Secretory HOMA	189.4±149.8	186.4±192.8	250.7±405.9	*NS*	269.9±181.8	248.5±151.4	277.6±214.5	*NS*

Results are presented as mean ± standard deviation (SD). Groups of data were compared using a one-way ANOVA test. Differences were considered significant at $p<0.05$. *GINR: Glucose insulin Ratio; HOMA B%= Beta Cell Function; HOMA S%= Beta Cell Sensitivity; HOMA-IR: Homeostasis Model of Assessment - Insulin Resistance; QUICKI= Quantitative Insulin Sensitivity Check Index; Secretory HOMA: Secretory Homeostasis Model of Assessment. SLC22A1 codon GG- wild-type homozygote; GA, heterozygote variant; AA, homozygote variant. NS= Not Significant.*

Table 3.40: Distributions of Lipidemic, ALT, and Creatinine Status according to SLC22A1 Genotype in the Study Subjects

Variables	*Study Subjects*							
	Diabetes (n=371)			*p value*	*Control (n=326)*			*p value*
	GG *(n=27)*	**GA** *(n=215)*	***AA*** *(n=129)*		**GG** *(n=89)*	**GA** *(n=137)*	***AA*** *(n=100)*	
Triglycerides (mg/dL)	147±64	155±82	147±79	*NS*	135±54	134±42	131±48	*NS*
Total cholesterol (mg/dL)	187±54	161±49	169±59	*NS*	162±34	175±34	176±29	*NS*
HDL-Cholesterol (mg/dl)	38±9	41±11	40±10	*NS*	49±14	51±15	52±14	*NS*
LDL-cholesterol (mg/dL)	136±41	102±41	111±53	*NS*	88±37	98±32	96±30	*NS*
TC: HDL-C	5.4±2.1	4.3±1.9	4.8±4.5	*NS*	3.6±1.3	3.7±1.2	3.6±1.0	*NS*
ALT (U/L)	27±20	28±14	31±18	*NS*	28±6	30±12	29±6	*NS*
Creatinine (mg/dL)	0.89±0.10	0.93±0.12	0.92±0.12	*NS*	0.95±0.11	0.94±0.12	0.92±0.11	*NS*

Results are presented as mean ± standard deviation (SD). Groups of data were compared using a one-way ANOVA test. Differences were considered significant at $p<0.05$. ALT Alanine Amino Transferase, Total cholesterol: HDL-cholesterol ratio SLC22A1 codon GG- wild-type homozygote; GA, heterozygote variant; AA, homozygote variant. *NS= Not Significant*.

3.7.12 Univariate Logistic Regression Analysis for Risk Factors of Diabetes with SLC22A1 Genotype in Diabetic Subjects

To estimate the association of various clinical factors and diabetes, univariate logistic regression models were performed with gender, BMI, family history of diabetes, glucose, HbA1C%, insulin, HOMA B%, HOMA S%, HOMA IR, triglycerides, total cholesterol and HDL-Cholesterol; and SNP of SLC22A1 gene (**Table 3.41**). The univariate logistic analyses indicated that family history of diabetes ($p<0.01$), BMI ($p<0.05$), glucose level ($p<0.001$), HbA1C% ($p<0.001$), HOMA B% ($p<0.05$), and total cholesterol ($p<0.05$) were significantly associated with diabetes. In diabetic subjects with SLC22A1 (G>A) and (GA+AA) variant, OR for diabetes were 0.578 (95% CI: 0.20 to 1.68, p=NS) and 1.705 (95% CI: 0.23 to 12.43, p= NS) respectively.

Table 3.41: Univariate Logistic Regression Analysis for Risk Factors of Diabetes with SLC22A1 Genotype in Diabetic Subjects

Variables	B	SE	*p value*	OR	95% CI
Gender	0.448	0.468	*NS*	1.565	0.62-3.92
Family History	-1.451	0.485	***<0.01***	0.234	0.09-0.61
BMI (Kg/m^2)	0.162	0.068	***<0.05***	1.175	1.03-1.34
Glucose (mM/L)	-3.194	0.893	***<0.001***	0.041	0.01-0.24
HbA1c (%)	-3.532	0.516	***<0.001***	0.029	0.01-0.08
Insulin (µU/L)	-0.203	0.147	*NS*	0.816	0.61-1.09
HOMA B%	-0.026	0.013	***<0.05***	0.974	0.95-1.00
HOMA S%	-0.006	0.012	*NS*	0.994	0.97-1.02
HOMA IR	1.833	1.329	*NS*	6.251	0.46-84.6
Triglycerides (mg/dL)	0.001	0.005	*NS*	1.001	0.99-1.01
T. Cholesterol (mg/dL)	0.013	0.007	***<0.05***	1.014	1.00-1.03
HDL-C (mg/dL)	0.066	0.024	*NS*	1.068	1.02-1.12
SLC22A1 (G>A)	-0.548	0.546	*NS*	0.578	0.20-1.68
SLC22A1 genotype (GA+AA)	0.534	1.013	*NS*	**1.705**	0.23-12.43

The reference category was GG variants group. *NS=Not Significant*.

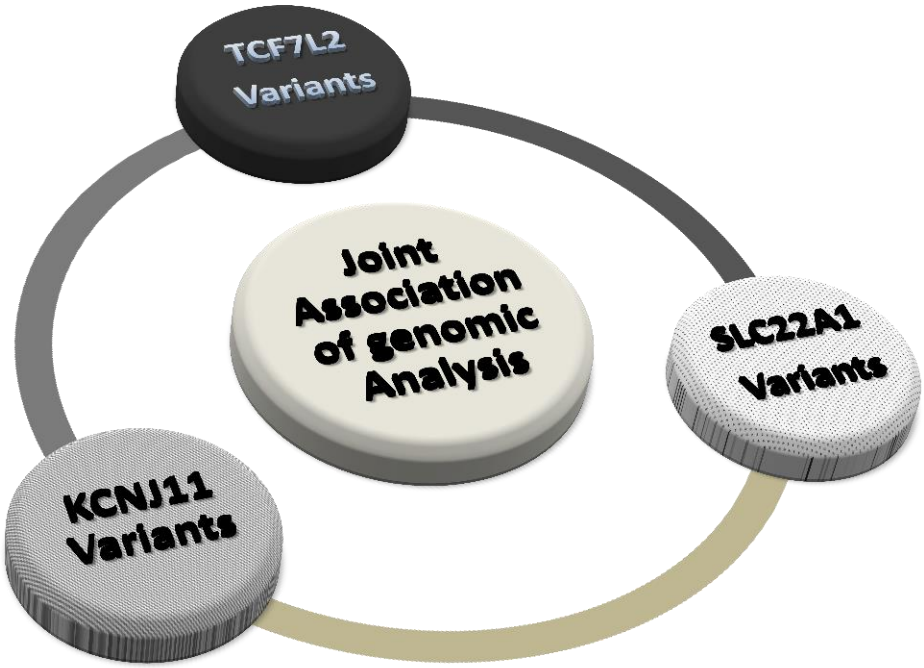
3.7.13 Multiple Logistic Regression Analysis for Risk Factors of Diabetes with SLC22A1 Genotype in Diabetic Subjects

Multiple logistic regression analysis demonstrated that the genetic variant of SLC22A1 remained significantly different ($p<0.001$) between diabetes and control after adjustment for potential confounders for genotype analysis **(Table 3.42).** After adjustment for confounding factors, in subjects with SLC22A1 (G/A) and genetic variants (GA+AA), the OR for diabetes were 1.217 (95% CI: 0.868 to 1.705) and 0.159 (95% CI: 0.082 to 0.308) respectively.

Table 3.42: Multiple Logistic Regression Analysis for Risk Factors of Diabetes with SLC22A1 Genotype in Diabetic Subjects

Variables	B	S E	*p value*	OR	(95% CI)
SLC22A1 allele (G>A)	0.196	0.172	*NS*	**1.217**	0.868-1.705
SLC22A1 Genotype (GA+AA)	-1.839	0.337	***<0.001***	0.159	0.082-0.308

Adjust with gender, family history of diabetes, BMI, glucose, HbA1C%, insulin, HOMA B%, HOMA S%, HOMA IR, triglycerides, total cholesterol, and HDL-cholesterol respectively. *NS=Not significant*.


TCF7L2
Variants
Joint
Association
of genomic
Analysis
SLC22A1
Variants
KCNJ11
Variants

3.8 Associations among KCNJ11, TCF7L2 and SLC22A1 Genotypes with Diabetic Risk

Table 3.43 presented pairwise joint associations of KCNJ11 (E^K), TCF7L2 (G^T), and SLC22A1 (G^A) genotypes with diabetic risk.

A significant pairwise joint association was found between KCNJ11 and TCF7L2 gene variants. The odd ratios of combined variants of EK/GT [OR (95% CI); 3.15 (1.85-5.34); p<0.001] and EK/TT [OR (95% CI); 14.33 (3.20-64.25); p<0.001] were found significantly higher in diabetic patients compared to controls when EE/GG was considered as reference group respectively. The odd ratios of combined variants of the KCNJ11 and SLC22A1 genes, EK/GA [OR (95% CI); 10.56 (5.33-20.91), p<0.001], EK/AA [OR (95% CI); 7.17 (3.47-14.82), p<0.001], KK/GA [OR (95% CI); 28.0 (3.19-245.4), p<0.001] and KK/AA [OR (95% CI); 12.00 (2.20-65.54), p<0.01] were significantly higher in diabetic group compared to control when combined variant EE/GG was considered as reference group respectively. A significant joint association was also observed between TCF7L2 and SLC22A1 genes in combined variants of GT/GA (p<0.001), GT/AA (p<0.001), TT/GA (p<0.001), and TT/AA (p<0.01) in consideration with GG/GG variants as a reference group.

Triple associations were found among KCNJ11, TCF7L2 and SLC22A1 genes. The odd ratios of combined variants of the KCNJ11, TCF7L2 and SLC22A1 genes in EK/GT/GA [OR (95% CI); 5.60 (2.20-14.25), p<0.001], EK/GT/AA [OR (95% CI); 10.82 (3.45-33.94), p<0.001], EK/TT/GA [OR (95% CI); 19.09 (3.64-100.1), p<0.001]; and EK/TT/AA & KK/TT/GA [OR (95% CI); 18.48 (0.83-412.70), p<0.05] variants were significantly higher in diabetic group than that of control, when EE/GG/GG variants used as reference.

Table 3.43: Joint Association of KCNJ11, TCF7L2 and SLC22A1 Genotypes for Risk of Diabetes in the Study Subjects

Joint Association of Genotypes	Study Subjects		OR (95% CI)	*p value*
	Diabetic	Control		
KCNJ11/ TCF7L2	**(n=168)**	**(n=248)**		
EE/GG	105	215	**1 Reference**	**---**
EK/GT	43	28	3.15 (1.85-5.34)	***<0.001***
EK/TT	14	2	14.33 (3.20-64-25)	***<0.001***
KK/GT	4	3	2.73 (0.60-12.43)	*NS*
KK/TT	2	0	10.21 (0.49-214.80)	*NS*
KCNJ11/ SLC22A1	**(n=175)**	**(n=128)**		
EE/GG	15	60	**1 Reference**	**---**
EK/GA	95	36	10.56 (5.33-20.91)	***<0.001***
EK/AA	52	29	7.17 (3.47-14.82)	***<0.001***
KK/GA	7	1	28.0 (3.19-245.4)	***<0.001***
KK/AA	6	2	12.00 (2.20-65.54)	***<0.01***
TCF7L2 / SLC22A1	**(n=163)**	**(n=143)**		
GG/GG	17	61	**1 Reference**	**---**
GT/GA	59	49	4.32 (2.24-8.34)	***<0.001***
GT/AA	54	23	8.43 (4.07-17.42)	***<0.001***
TT/GA	26	7	13.33 (4.94-35.97)	***<0.001***
TT/AA	7	3	8.37 (1.95-35.90)	***<0.01***
KCNJ11/ TCF7L2 / SLC22A1	**(n=67)**	**(n=67)**		
EE/GG/GG	11	42	**1 Reference**	**---**
EK/GT/GA	22	15	5.60 (2.20-14.25)	***<0.001***
EK/GT/AA	17	6	10.82 (3.45-33.94)	***<0.001***
EK/TT/GA	10	2	19.09 (3.64-100.1)	***<0.001***
EK/TT/AA	2	0	18.48 (0.83-412.70)	***<0.05***
KK/GT/GA	1	1	3.82 (0.22-66.07)	*NS*
KK/GT/AA	2	1	7.64 (0.63-92.21)	*NS*
KK/TT/GA	2	0	18.48 (0.83-412.7)	***<0.05***
KK/TT/AA	0	0	3.70 (0.07-196.70)	*NS*

Results are expressed as number (percentage). Fisher's exact test was performed to explore the association of case and control group for the genotype frequencies. $p<0.05$ was taken as the level of significance. Odds ratios (OR) and 95% confidence interval (95% CI). KCNJ11 genotypes, Wild type homozygote, E23E; heterozygote variants, E23K; homozygote variants, K23K. TCF7L2 genotypes, Wild type homozygote, GG; heterozygote variants, GT; homozygote Variants, TT.SLC22A1 codon GG- wild-type homozygote; GA, heterozygote variant; AA, homozygote variant. *NS=Not Significant*.

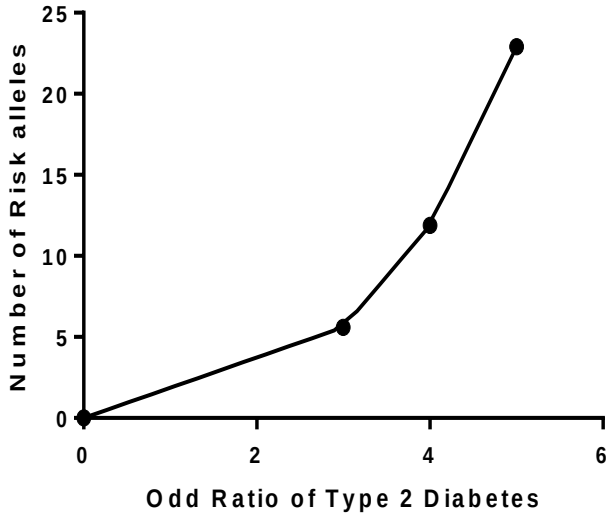
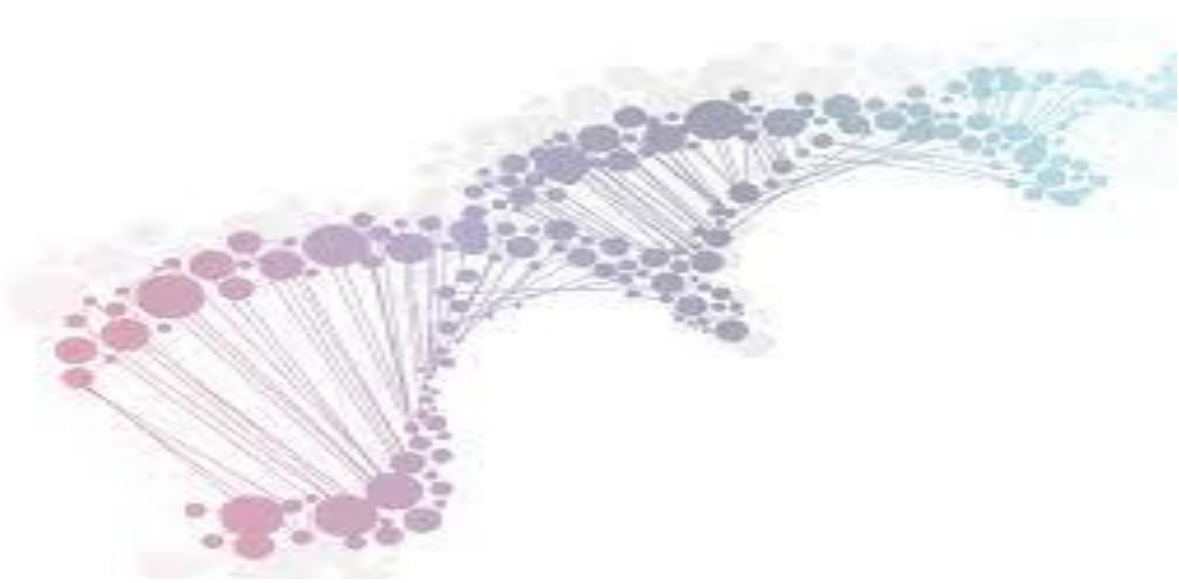


Figure 3.22: Odd Ratio of Combined Variants of KCNJ11, TCF7L2 and SLC22A1 Genes with Risk of Diabetes

Graphic presentation of ORs and corresponding 95% CI for type 2 diabetes estimated from 371 type 2 diabetic and 326 control subjects categorized into five groups (n= 0, 3, 4, 5, and 6) according to the number of risk alleles. Polymorphic "K" allele of the *KCNJ11* E23K, "T" allele of the TCF7L2, and "A" allele of the SLC22A1 gene were considered as type 2 diabetes risk alleles. The group with no risk alleles comprised 53 subjects (11 type 2 diabetic patients and 42 control subjects, respectively); the group with risk three allele comprised 37 subjects (22 T2DM and 15 control); the group with risk four alleles comprised 37 subjects (28 T2DM and nine control); the group with risk five alleles comprised seven subjects (6 T2DM and one control); the group with risk six alleles comprised no subjects (no subject in both group). The ORs for each group were estimated relative to the group with three risk alleles by logistic regression. It is suggested that OR of EK/TT/GA, EK/TT/AA, and KK/TT/GA alleles showed the maximum risk in T2DM subjects, respectively.

Chapter 4
Discussion and Conclusions



4. Discussion and Conclusion

4.1 Discussion

Diabetes has become a widespread epidemic, primarily due to the increasing prevalence and incidence of type 2 diabetes. A meta-analysis in Bangladesh showed that the prevalence of diabetes among adults had increased substantially, from 4% in 1995-2000 to 5% in 2001-2005 and then to 9% in 2006-2010, respectively (Shamima et al., 2014; Khan, 1998).

In this case-control study, smoking status was higher in the diabetic group than control, but this value is not statistically significant (**Table 3.1**). Although a positive association ($p<0.001$) between cigarette smoking and the development of diabetes was reported by Xie et al. (2009). In an InterAct case-cohort study, Scott et al. (2013) found a positive association of family history of diabetes among different family members with the incidence of T2DM. A significant association ($p<0.05$) of a family history of diabetes with the incidence of T2DM was also found in this study (**Table 3.1**).

One of the major risk factors for T2DM is obesity. Clinical evidence indicates a positive association of diabetes with central obesity than general obesity (Kamath et al., 2011). A strong positive association between obesity and diabetes was also reported by Awasthi et al. (2017). In this study, a significant association was found between diabetes and body fat percentage ($p<0.05$) and WHR ($p<0.001$) in diabetic than that of control subjects **(Table 3.2)**. It has been reported that a 20 mm Hg higher SBP than usual was linked to 58% higher risk of new-onset diabetes, and a 10 mm Hg higher DBP than usual was linked to 52% higher risk of developing diabetes (Emdin et al., 2015). In this study, a significant positive association ($p<0.001$) of DBP and SBP with diabetes was also found in diabetic subjects compared to control **(Table 3.2)**.

In the present study, a positive association of glycemic and insulinemic status with diabetes was found. Baseline data showed that glucose and HbA1C% of diabetic subjects were significantly higher than that of control. On the other hand, HOMA B%; HOMA S%, QUICKI, and secretory HOMA were significantly lower, whereas insulin and insulin

resistance HOMA IR was significantly higher in diabetic subjects than control (**Table 3.3**). Unnikrishnan et al. (2014) also found a positive association of characteristic metabolic profile of diabetes with high insulin level, a greater degree of insulin resistance, and a higher prevalence of diabetes. In a cross-sectional study, Abdul et al. (2010) reported that the normal glycemic status is related to lower risk of type 2 diabetes due to higher insulin sensitivity and better β-cell function. It has been reported that insulin resistance and insulin secretory dysfunction are independently and interactively related to type 2 diabetes risk in adults (Weyer et al., 1999; Haffner et al., 1995; Lillioja et al., 1993). Specifically, the inability of the β-cell to compensate for insulin resistance is a primary determinant of type 2 diabetes (Weyer et al., 1999; Bergman et al., 2002). The above reports supported the findings of the present study (**Table 3.3**).

There is a strong association between type 2 diabetes and dyslipidemia. In general, the improved glycemic control in diabetes causes favorable effects on lipoprotein levels, by reducing the cholesterol and triglyceride levels through the decreased circulation of very-low-density lipoprotein (VLDL) and by increased catabolism of LDL through reduced glycation and upregulation of LDL receptors (Pietri et al., 1983; Tames et al., 1982). In the present study, the TG ($p<0.01$) and LDL ($p<0.001$) were significantly higher; and HDL ($p<0.001$) and cholesterol & HDL ratio ($p<0.001$) were significantly lower in diabetic subjects compared to control respectively which was inconsistent with the finding of others (Pietri et al., 1983; Tames et al., 1982) **(Table 3.4).**

Zhang et al. (2015) reported that with an increasing family history of diabetes, the fasting glucose and insulin, TC, TG, and LDL-cholesterol levels were showed a significantly increasing trend. But in this study, no significant difference was found in glycemic, insulinemic, and lipidemic status between diabetic subjects with a positive family history of diabetes and that of without family history of diabetes. Whereas, a significant increase of HbA1C, insulin, and HOMA IR was found in control subjects with a positive family history of diabetes than control subjects without a family history of diabetes. The results suggested that the positive family history of diabetes may be a predisposition factor for diabetes in control subjects **(Table 3.5)**.

Several studies reported a correlation between HbA1C and lipid profile. Some of these have shown that glycemic status has a significant correlation with lipid profile (Gligor et al., 2011; Lavanya et al., 2017). Besides maintaining long term glycemic control, HbA1c can also deliver valuable supplementary information about the extent of circulating lipids (Gligor et al., 2011). It was also reported by Lavanya et al. (2017) that worse glycemic status (HbA1C >9%) possessed significantly high values of TC, TG, LDL-C, glucose except HDL-C as compared to patients having poor (HbA1C 6-9%) and good (HbA1C<6%) glycemic control, In the present study, no significant difference was found between HbA1C and lipidemic status of diabetes subjects, the results were contradictory to the findings of others **(Table 3.6)**.

As shown in **Table 3.7**, the fasting insulin, HOMA B%, and HOMA IR were significantly higher; and GINR and HOMA S% were lower in graduate than undergraduate diabetic subjects in graduate diabetic subjects. Insulin resistance may be due to uncontrolled and unhealthy food intake. LDL cholesterol level was significantly higher ($p<0.05$) in undergraduate diabetic subjects than graduates, which may be due to high carbohydrate intake in their diet being unaware of the harmful effect of excessive carbohydrate intake. A study was done on the Indian population also reported that diabetes awareness, treatment, and glycemic status were poor in low educated diabetic subjects (Gupta et al., 2015). The present study revealed that diabetes has a high prevalence in the low educational population, and the result is consistent with the findings of Gupta et al. (2015) **(Table 3.7).**

The effect of urbanization is an epidemiological transition towards increasing rates of obesity and non-communicable diseases (NCDs), including T2DM. Wai et al. (2018) and Hussain et al. (2005) reported that the prevalence of DM was significantly higher in urban than in rural areas in the Yangon Region. Whereas, in the present study, no association was found on diabetogenic parameters (glycemic, insulinemic and lipidemic status) with the area of residence in diabetic subjects; but in control subjects, HbA1C and TC were significantly higher in urban population, HOMA B% and secretory HOMA were lower and HOMA S% ($p<0.05$) is higher in semi-urban subjects than that of rural, which may

indicate higher early predisposition factors of diabetes in a residence of a semi-urban and urban area **(Table 3.8)**.

Many studies have compared and correlated glycemic control markers with lipid profiles in diabetes. Reddy et al. (2014) and Thambiah et al. (2016) reported that glucose had a significant positive correlation with TC, TG, LDL-C, and a negative correlation with HDL-C. In this study, a positive correlation was also found between fasting glucose levels with TC, TG, and LDL-C in diabetic subjects, respectively **(Table 3.9)**. Many other researchers also found a positive correlation with glucose and lipidemic status (Sheikhpour et al., 2013; Sapkota and Thapa, 2017). Reddy et al. (2014) reported that HbA1c had a positive correlation with TC, TG, and LDL, whereas Mahato et al. (2011) reported a positive correlation between HbA1c with TC and LDL-C. Further, a positive correlation of HbA1c level with TC, LDL, and TG in diabetic patients was reported by several studies (Erciyas et al., 2004; Andersen et al., 1983; Ohta et al., 1998). In this study, only a positive correlation was found between HbA1C and TG in the diabetic group, but not with TC, HDL-C, and LDL-C **(Table 3.9).**

Longitudinal studies have revealed that both defects in the early phase of insulin secretion and insulin sensitivity can predict overt diabetes (Lundgren et al., 2009; Weyer et al., 1999). Progressive deterioration in early insulin secretion was connected with the transition from normal glucose tolerance to diabetes (Weyer et al., 1999). Overt type 2 diabetes can also be characterized by deteriorating of insulin secretion with time. This gradual decrease of insulin secretion, despite unchanged insulin sensitivity, has been shown by longitudinal cohort studies. United Kingdom Prospective Diabetes Study (UKPDS) showed that in the control group, the residual insulin secretory capacity was reduced by 50% at the time of diabetes diagnosis. After six years, a further 15% reduced (UKPDS, 1995). A significant positive correlation coefficient between insulin with HOMA B%, HOMA IR, and Secr HOMA was found, and a significant negative correlation coefficient was found with HOMA S% and QUICKY, respectively. On the other hand, a significant negative correlation was found in glucose and HbA1C individually with HOMA B%, HOMA S%, QUICKY, and Secr HOMA, respectively. Our result correlates with the findings of UKPDS (1995) **(Table 3.9).**

To identify and establish the genes responsible for the development and risk of type 2 diabetes, numerous studies have been conducted. Using candidate gene analysis and GWAS, investigators have identified several potential susceptible genes for T2DM. In this study, a case-control investigation was carried out to determine the association of KCNJ11, TCF7L2, SLC22A1 gene with the development of T2DM in a cohort in the population of Bangladesh. To the best of our knowledge, this study is the first attempt to find the association of genetic polymorphisms of KCNJ11, TCF7L2, SLC22A1 genes with an increased risk of T2DM in the Bangladeshi population.

The protein encoded by KCNJ11 plays a vital role in insulin secretion, making it a potential susceptible gene for T2DM. The association between KCNJ11-E23K polymorphism and the incidence of T2DM is controversial. Some studies have reported no association of KCNJ11 with diabetes in the Chinese Han and Caucasians population (Gloyn et al., 2001; Van et al., 2005). Keshavarz et al. (2014) and Wang et al. (2011) had also failed to found any impact of KCNJ11 E23K on the risks of T2DM in Iranian and Chinese Han populations, respectively. The results were similar to those found by meta-analysis in a European population-based study (Van et al., 2005). On the other hand, a significant association of KCNJ11 E23K with the incidence of T2DM has been reported by others (Schwanstecher et al., 2002; Wang et al., 2009; Zhou et al., 2009). Rastegari et al. (2015) reported that both the KK homozygous genotype and carriers of K allele of KCNJ11 have more risk ($p<0.005$ and $p=0.05$ respectively) for T2DM. Similar results were also found for the Russian and Chinese Han population (Chistiakov et al., 2008; Li et al., 2013). Asaf et al. (2013) reported that the inheritance of the K allele predisposes for T2DM. In this study, the frequency of heterozygous EK ($p<0.001$) and homozygous KK ($p<0.05$) genotype were higher in diabetic subjects compared to homozygous EE, which indicated a higher risk for T2DM. Whereas the carrier of K allele was significantly higher ($p<0.01$) in diabetic subjects compared to the E allele. The results demonstrated that the allelic and genotypic distribution of KCNJ11 E23K polymorphism is positively associated with T2DM in the Bangladeshi population (**Table 3.10**).

The reported prevalence of K23 allele was 41% in Chinese Han (Wang et al., 2009), 37% in the UK (Gloyn et al., 2001), 39% in Danish (Nielsen et al., 2003), 36% in a Japanese (Sakamoto et al., 2007), 39% in French (Vaxillaire et al., 2007) and 39.2% in Caucasians diabetic population of European origin (Nikola et al., 2009). Further, Ezzidi et al. (2009) reported a slightly lower E23K allele frequency (30.5%) in an Arabic population from Tunisia. In the present population-based study, 43% frequency of heterozygous E23K was found in Bangladesh (**Table 3.10**), close to the Chinese Han population.

In this study, a significant association of E23K genotypes with diabetes was found in male ($p<0.05$) and female ($p<0.001$) diabetic subjects, when the E23E genotype was considered as the reference group. The results indicated that the E23K genotype carries a higher risk for the incidence of diabetes in both males and females (**Table 3.11**). Contradictory to our findings, no significant difference was found for E23K and K23K genotype with diabetic male and female subjects by other studies (Yi et al., 2014; Jung et al., 2012; Pietrzak-Nowacka et al., 2013).

Raden et al. (2015) suggested a higher risk of developing T2DM for individuals having K allele and a family history of diabetes. They found a significantly higher ($p=0.001$) frequency of K allele in individuals with a family history of diabetes (68%) than without family history. In this study, the frequency of E23K variants of KCNJ11 genotypes was significantly higher ($p<0.001$) compared to E23E variants in diabetic subjects with a positive family history of diabetes. Whereas, E23K and K23K variants without a family history of diabetes showed no significant association **(Table 3.12).** The finding of our study is in accordance with the previous research. Nagaraja et al. (2014) reported a significant association of KCNJ11 (K23K) genotype with early age of disease onset ($p<0.05$) in South Indian T2DM subjects. However, we did not found any significant association on the onset of diabetes with KCNJ11 genotypes **(Table 3.13)**.

In the present study, the frequency of E23K and K23K variants of KCNJ11 genotypes were not associated with diabetic subjects residing in a rural area. Still, a significant association was found in the E23K variant of diabetic residents living in semi-urban

($p<0.05$) and urban ($p<0.001$) areas, respectively, than that of control subjects. The result indicated that the E23K genotype of KCNJ11 might be a risk factor for the semi-urban and urban population **(Table 3.16)**.

To explore the possible mechanism of KCNJ11 variants on the susceptibility to T2DM, the association of the KCNJ11 polymorphisms with T2DM in relation to glucose, HbA1c, TC, TG, HDL, LDL, ALT, and Creatinine, etc. were investigated. No significant association was found for glycemic, insulinemic, lipid profiles, ALT, and Creatinine status with KCNJ11 genotypes **(Table 3.17 and Table 3.18)**. Similar results were also reported by other studies (Ayat et al., 2016; Pietrzak-Nowacka et al., 2013). On the other hand, Nagaraja et al. (2014) reported that in the South Indian population, the KCNJ11 rs1800467 G/G genotype showed a significant increase of TC level ($p=0.01$) in T2DM subjects when compared to other variants. Khaled et al. (2014) also reported that FBG levels were higher in subjects with the K23K genotype than that of the E23K and E23E genotypes and no significant differences were observed with TC, TG, and HDL-C. A significantly lower level of fasting plasma insulin was found in Taiwan childhood and adolescent diabetic subjects with K23K genotype than that of without the K23K genotype. In a population-based Inter 99 cohort study using Danish Caucasian population, Sara et al. (2005) also reported similar results.

By univariate logistic analyses, we found that BMI ($p<0.01$), glucose ($p<0.001$), TG ($p<0.05$), TC ($p<0.01$), and HDL-C ($p<0.001$) were significantly associated with diabetes risk. ~~S~~subjects with KCNJ11 (E23K) genotype showed a 1.76-fold higher risk for the incidence of diabetes **(Table 3.19)**. Khaled et al. (2014) reported that the KCNJ11 E23K genotype was independently related to fasting plasma glucose levels. The fasting plasma glucose level only explained 65% increase of the E23K variant. Our results are similar to the results reported by Khaled et al. (2014).

The TCF7L2 gene-encoded transcription factor involved in the Wnt signaling pathway plays a critical role in cell development and regulatory mechanisms (Prestwich and Macdougald, 2007). Smith (2007) suggested an important role of TCF7L2 polymorphisms

in the susceptibility of T2DM by indirectly altering glucagon-like peptide 1 (GLP-1) levels, as GLP-1 and insulin plays a significant role in blood glucose homeostasis.

Several studies revealed that the risk allele frequency for the SNPs T of rs12255372 (G/T) (Chandak et al., 2007; Uma et al., 2013; Jinjin et al., 2013), T of rs7903146 (C/T) (Kimber et al., 2007; Chandak et al., 2007; Uma et al., 2013), T of rs4506565 (A/T) (Chandak et al., 2007) and C of rs11196205 (G/C) (Uma et al., 2013) of TCF7L2 gene were strongly associated with risk of diabetes. In the present study, we had also found a significant association in the genotype category GG vs. TT ($p<0.01$) and GG vs. GT+TT ($p<0.05$) of TCF7L2 (rs12255372) gene in diabetic subjects and the allele frequency for minor "T" allele was significantly ($p<0.05$) high in diabetic subjects when "G" allele was considered as the reference group **(Table 3.21)**. Whereas, Ren et al. (2008) reported contradictory result in rs12255372 of the TCF7L2 genotype in Chinese Han populations. Chang et al. (2007) identified an association of the rs290487 polymorphism of the TCF7L2 gene in a Chinese population but without any association of T2DM with the rs7904136 and rs12255372.

Zhiqiang et al. (2017) reported that the distribution of variant CC vs. TT ($p<0.001$) and the dominant model (CC vs. CT+TT, $p<0.001$) both in rs7903146 polymorphism of TCF7L2 gene were significantly higher in male diabetic subjects than that of male control subjects respectively, which indicates that the risk of T2DM is higher in men carrying T allele. In this study, also a significant ($p <0.05$) frequency difference was found in TT genotype compared to the GG genotype in diabetic male subjects. This indicates that TT variants had a high risk for diabetes's predisposition of diabetes in Bangladeshi male subjects when the GG variant was considered reference, which is supported by Zhiqiang et al. (2017) (**Table 3.22)**.

A significant association ($p<0.05$) of positive family history of diabetes with the TCF7L2 TT variant was found in this study (**Table 3.23**). On the other hand, Marilyn et al. (2019) reported no significant association between positive family history of diabetes and variants of TCF7L2 genotype. In the present study, the frequency of GT and TT variants of TCF7L2 genotypes were found to have a significant association with the residence of

semi-urban ($p<0.05$) and urban ($p<0.05$) diabetic subjects than control, which indicates that TCF7L2 genotype may be a risk factor for the semi-urban and urban population **(Table 3.27)**.

The relative role of defects in insulin resistance and insulin secretion to type 2 diabetes's pathogenesis remains controversial (Groop et al., 2000; Chiasson and Rabasa-Lhoret, 2004). Richa et al. (2006) were reported that individuals TT homozygous for the rs7903146 risk allele have a significant reduction in insulinogenic and insulin disposition index. They also suggested that the polymorphism influences the early insulin response to glucose and could affect the capacity of the cells to secrete insulin. In another study, Wang et al. (2007) reported that the "T" allele SNP of TCF7L2 was associated with decreased first-phase insulin release in an intravenous glucose tolerance test (IVGTT) in non-diabetic offspring of type 2 diabetic subjects. Results of our study also suggested the decrease of insulinemic parameters (fasting insulin level, HOMA B%, secretory HOMA, and HOMA IR) and a significant ($p<0.05$) increase of QUICKI in T allele SNP of TCF7L2 genotype in diabetic subjects, which purported the previous two reports (**Table 3.28**). Similarly, Habiba et al. (2015) reported that there was no association between lipidemic status with CT+TT (TCF7L2 rs10885409) genotype of diabetic subjects in the Emirati Population (**Table 3.29**). No association was found in ALT and creatinine levels with TCF7L2 genotypes in the study subjects.

In univariate logistic regression analyses with BMI, family history of diabetes, glucose, HOMA B%, TC, and HDL-C considering heterozygous GT variant of TCF7L2 as the dependent variable; and multivariate logistic regression analyses with adjustment of some confounding factors (gender, BMI, BP, family history of diabetes, glucose, insulin, HOMA B%, HOMA S%, HOMA IR, TC, TG, HDL-C) for diabetes, the heterozygous GT variant of TCF7L2 was significantly associated with a high risk of diabetes **(Table 3.30 and Table 3.31**). A similar result was also reported by Zhiqiang et al. (2017). The rs7903146 polymorphism was associated with the risk of developing T2DM in the dominant model after adjustment for potential confounders, individuals with CT+TT genotype in rs7903146 of the TCF7L2 gene than individuals with CC genotype.

Several studies reported the genotypic distributions and diabetic risk assessment of the SLC22A1 gene in T2DM. Very low genotypic frequency of wild-type variant GG in SLC22A1 gene in T2DM subjects were reported, such as 5.0% and 5.81% wild type genotype GG in Javanese-Indonesian (Vitarani et al., 2019; Ningrum et al., 2019), 14.63% in Latvian (Tarasova et al., 2012); 14.81% in Slovenian (Klen et al., 2014) and 6.57% in Chinese Han population (Zhou et al., 2015). The non-existence of GG wild type variant was similar to the findings reported for Japanese and Asian-American people (Itoda et al., 2004; Shu et al., 2003). In the present study, 7.3% of wild type variant (GG) was found in T2DM subjects compared to control (27.3%), and the allele frequency of "G" was significantly ($p<0.01$) lower than "A" allele, which supported the results of above studies (**Table 3.32)**. Meanwhile, among African-American and European-American populations, the mutant allele "A" frequencies were 2.9% and 18.5%, respectively (Shu et al., 2003). Studies involving in Iranian T2DM (Mahrooz et al., 2015), Caucasian healthy (Kerb et al., 2002; Tzvetkov et al., 2009), and Danish subjects (Christensen et al., 2011), the frequency of wild-type variant was higher than that of heterozygous and homozygous mutant variants, which suggested a contradictory result with the present study.

Ningrum et al. (2019) suggested no GG variant (wild type) found in the SLC22A1 Met420del gene, and only 4% of the subjects had the GA variant. The highest proportion of mutant allele A (>95%) in both male and female subjects was found, and even in the male patient, 100% of them had typed 'a' mutant allele (Ningrum et al., 2019). Similarly, we also found a higher mutant allele of (GA+AA) both in males and females (93% and 92.3%, respectively) **(Table 3.33).**

A study by Koshy et al. (2016) with OCT1 rs122083571 polymorphism (C/T allele) found that glucose, insulin, TC, and LDL-C levels were significantly high in diabetic subjects with polymorphic A allele when compared to that of the G allele containing subjects. In this study, no associations were found in glucose, HbA1C%, and insulin levels with genotypes. But HOMA IR was decreased in the AA variant than GG and GA variants in diabetic subjects, but this result was not statistically significant (**Table 3.39**). No association was found in lipid profiles, ALT, and creatinine level with SLC22A1 genotypes in the study subjects.

In the present study, the univariate logistic analyses indicated that family history of diabetes, BMI, glucose, HbA1c%, HOMA B%, and TC were significantly associated with the incidence of diabetes (**Table 3.41)**. On the other hand, after adjusting the confounding factors (gender, family history of diabetes, BMI, glucose, HbA1C%, insulin, HOMA B%, HOMA S%, HOMA IR, TG, TC, and HDL-C), multiple logistic regression demonstrated that in diabetic subjects with dominant model GA+AA variants have significantly higher risk for the predisposition of diabetes (**Table 3.42).** Laith et al. (2019) also reported that BMI and age at diagnosis of diabetes were significantly associated with glycemic control ($p<0.05$). Therefore, these covariate factors should be considered when treating patients with diabetes**.**

A joint association linkage analysis among KCNJ11, TCF7L2, and SLC22A1 genes suggested that the group with higher polymorphic risk alleles (EK/TT/GA, EK/TT/AA, and KK/TT/GA) showed the maximum risk in T2DM subjects, which indicates that the higher frequency of polymorphic combined variants is significantly associated with the increased risk of diabetes than that of less polymorphic combination variants (**Table 3.43).**

4.2 Conclusions

In conclusion, the genome analysis of KCNJ11, TCF7L2, and SLC22A1 genes revealed that the E23K and K23K variants of the KCNJ11 gene had shown 2.02 and 2.70 folds; TT and GT+TT variants of TCF7L2 gene has shown 2.94 and 1.45 folds; & GA and AA variants of SLC22A1 gene has shown 5.17 and 4.25 folds high risk for type 2 diabetes in Bangladeshi population. E23K variants of KCNJ11 gene showed 2.39 times high risk for female and 1.17 times the increased risk for male; TT variants of TCF7L2 has 3.24 folds high risk only for male; and in case of SLC22A1 gene, GA and AA variants has a high-risk factor for both male (OR of GA= 3.61 & AA=3.07) and female (OR of GA=7.47 & AA=5.66) in diabetic subjects. According to a family history of diabetes, E23K variants of KCNJ11 showed 2.64 folds, and TT variants of TCF7L2 showed 5.37 folds high risks for diabetes incidence with a positive family history of diabetes than without a family history of diabetes. Whereas GA and AA variants of SLC22A1 also have a higher risk for subjects (diabetes and control) with or without a family history of diabetes, respectively. Regarding residence, the E23K variant of the KCNJ11 genotype has been found to have a high risk for diabetes in semi-urban (OR 3.37) and urban (OR 2.14) population compared to rural. Whereas, GT and TT variants of the TCF7L2 gene; and GA and AA variants of the SLC22A1 gene showed a high risk for the predisposition of diabetes in semi-urban and urban populations than rural population. The joint association of three genomic studies reveals that subjects with 3, 4, and 5 allele variants of KCNJ11/TCF7L2/ SLC22A1 genotypes showed an increased risk of diabetes.

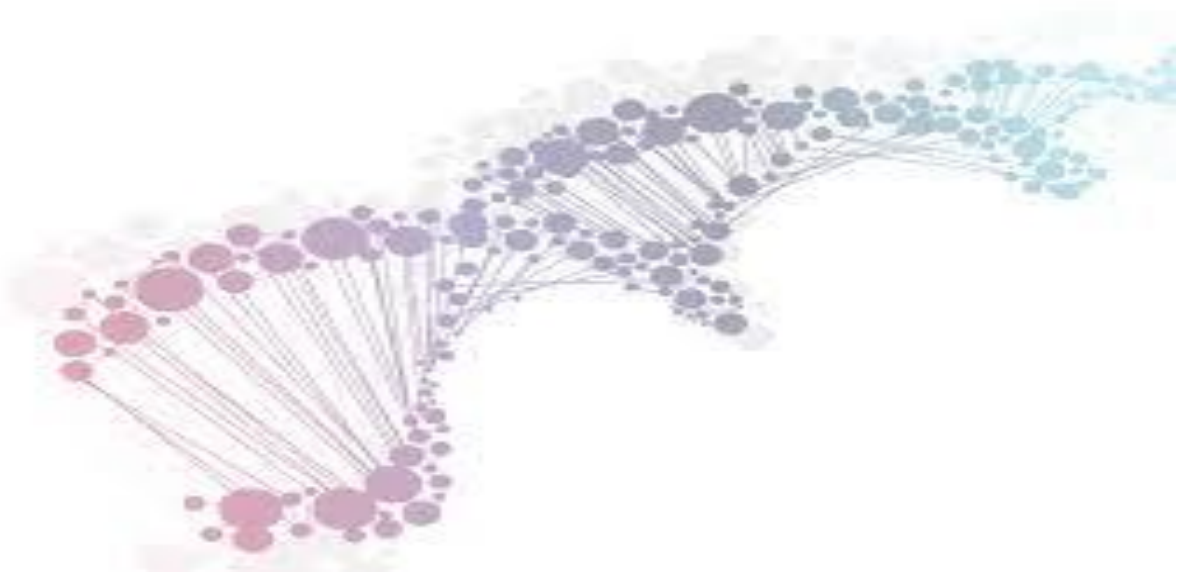
4.3 Limitations of the Study

The following limitations of the study may be considered for interpretation of the results:

- First, the study was a hospital-based case-control study. The T2DM cases and controls were recruited from the hospital so that a selection bias may occur.
- Second, since the study participants were recruited from Dhaka and outside of Dhaka, Bangladesh, so they are not representative of the Bangladeshi population as a whole.
- Third, the duration of the disease in the study subjects was not considered, so subjects with a more prolonged duration of diabetes could have reduced endogenous insulin production, meaning that individuals had different levels of endogenous insulin and subsequently led to bias in selecting subjects.
- Fourth, though OGTT is the best option to exclude diabetes and the gold standard for diabetes prediction. But, with such a large sample size (control, n= 327), it was not possible to measure OGTT with unavailable logistic support and the involvement of enormous cost. Therefore, instead of OGTT, diabetes was excluded by considering other biochemical parameters and ensuring that the controls are not taking any antidiabetic drugs without any other complications related to diabetes.

However, it is important to note that the present study is the first to investigate the association between the aforementioned KCNJ11/TCF7L2/SLC22A1 SNPs and T2DM in the Bangladeshi population.

Chapter 5
References



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Appendix I

বাংলাদেশ ডায়াবেটিক সমিতি
DIABETIC ASSOCIATION OF BANGLADESH

Memo No. BADAS-ERC/EC/13/00163 Date: June 19, 2014

To

Amrita Bhowmik
PhD Student
Biochemistry and Molecular Biology
University of Dhaka

Subject: Ethical Clearance

The ethical review committee (ERC) of the Diabetic Association of Bangladesh has approved your protocol on "**Association of Genetic Variation in TCF7L2, SLC22A1 and KCNJ11 Genes with Risk for Type 2 Diabetes in Bangladeshi Population.**"

(Dr. KMS Aziz)
Chairman
Ethical Review Committee

122 KAZI NAZRUL ISLAM AVENUE SHAHBAG DHAKA-1000 BANGLADESH PHONE : 8616641-50, 863-2 966 1511 FAX : 9677772, Web : www.dab-bd.org

Appendix II

Department of Biochemistry and Molecular Biology
University of Dhaka
Dhaka – 1000
Bangladesh

প্রাণরসায়ন ও অণুপ্রাণ বিজ্ঞান বিভাগ
ঢাকা বিশ্ববিদ্যালয়
ঢাকা - ১০০০
বাংলাদেশ

OFFICE OF ETHICAL REVIEW COMMITTEE
Department of Biochemistry and Molecular Biology
University of Dhaka, Bangladesh

January 12, 2017

BMBDU-ERC/EC/17/09

Ms. Amrita Bhowmik
Ph.D Student
Department of Biochemistry and Molecular Biology
University of Dhaka

Subject: Institutional Ethical Review Committee (ERC) Clearance

Dear Ms. Bhowmik,

With reference to your application on the above mentioned subject, this is to inform you that your Ph.D research proposal titled "**Association of Genetic Variation in TCF7L2, SLC22A1 and KCNJ11 Genes with Risk for Type 2 Diabetes in Bangladeshi Population**" has been reviewed and approved by the Departmental Ethical Review Committee in its 5th meeting held on 19th January, 2017.

You are requested to follow the Institutional Ethical Review Committee guidelines. Please note that failure to comply with the conditions of approval may result in withdrawal of ethical clearance approval of the proposal.

Expiry date and Expected submission date of thesis: November, 2019.

Prof. Dr. Yearul Kabir
Chairman
Departmental Ethical Review Committee

Telephone: 9661920-73 Ext.7637 Fax: 880-2-8615583 Internet: http://www.bmbudhaka.net

Appendix III

সন্মতিপত্র

গবেষনার বিষয়

TCF7L2, SLC22A1 এবং KCNJ11 জীনের পলিমরফিজমের কারনে বাংলাদেশে টইপ ২ ডায়াবেটিস হওয়ার প্রবনতা নির্ণয়।

প্রাণরসায়ণ ও অনুপ্রাণ বিজ্ঞান বিভাগ, ঢাকা বিশ্ববিদ্যালয়

পরীক্ষা সংক্রান্ত ব্যাখ্যা,

পৃথিবী এখন ডায়াবেটিসের মহামারীতে আক্রান্ত । বাংলাদেশের মত উন্নয়নশীল দেশেও এর প্রকপ দিনকে দিন বাড়ছে। বর্তমানে মূখেসেবনীয় অনেক ডায়াবেটিস নিয়ন্ত্রণকারী ঔষধ বাজারে সহজপ্রাপ্য। অনেক গবেষণায় দেখা গেছে যে, কিছু কিছু ঔষধের কার্যকারিতা সম্পর্কিত জীনের ভিন্নতা বা পলিমরফিজমের কারনে মূখেসেবনীয় ঔষধগুলো সঠিকভাবে ডায়াবেটিক নিয়ন্ত্রণে ভূমিকা রাখতে পারে না । এই গবেষণায় দেখা হবে যে, ডায়াবেটিক রোগীদের তিনটি জীন TCF7L2, SLC22A1 এবং KCNJ11 এর পলিমরফিজম ডায়াবেটিস হওয়ার ঝুকি বাড়ায় কিনা এবং ডায়াবেটিস নিয়ন্ত্রণে এইসব জীনের আদৌ কোন সম্পর্ক আছে কিনা।

আপনাকে এ গবেষণায় অংশগ্রহনে জন্য অনুরোধ জানাচ্ছি। আপনাকে অংশগ্রহনের প্রথম দিন বাছাই পর্বের পর ৮ মিলিলিটার রক্ত হাতের শিরা থেকে দিতে হবে । ৩ মিলিলিটার রক্ত আমরা সংরক্ষণ করবো জেনেটিক গবেষণার জন্য এবং ৫ মিলিলিটার রক্ত দিয়ে আপনার রক্তের গ্লুকোজ, কোলেষ্টেরল, ট্রাইগিণ্টসারাইড, এইচ ডি এল, এল ডি এল, ক্রিয়েটেনিন, এ এল টি এবং ইন্সুলিন এর মাত্রা নির্নয় করবো। উক্ত টেস্টগুলো আমরা আপনাকে বিনামূল্যে নির্নয় করে দেব। এতে অল্প ব্যথা লাগতে পারে তবে অন্য কোন জটিলতা দেখা দেবে না এবং স্বাস্থ্যগত কোন ঝুকিও থাকবে না। এই বিশেষ গবেষনায় অংশগ্রহণ করা বা না করার উপর বর্তমান ডায়াবেটিস চিকিৎসার কোন হেরফের হবে না। গবেষনায় অংশগ্রহণের জন্য রাজী হওয়ায় আপনাকে অভিনন্দন জানাচ্ছি।

অংশগ্রহণকারীর সন্মতি

আমি স্বজ্ঞানে এ পরীক্ষা সম্পর্কে বর্ণিত উপরোক্ত বিষয়াদি অবগত হলাম এবং আমাকে সেগুলো বুঝিয়ে দেওয়া হলো, এ পরীক্ষায় অংশগ্রহণের জন্য আমি পূর্ণ সমর্থন জ্ঞাপন করছি।

গবেষকের স্বাক্ষর
তারিখঃ

অংশগ্রহণকারীর স্বাক্ষর
তারিখঃ

Appendix IV

Questionnaires

Title: Association of genetic variation in TCF7L2, SLC22A1 and KCNJ11 genes with risk for Type 2 diabetes in Bangladeshi population

ID. AM- **Date:**

1. a. Name: _____

b. Father's / Husband's Name: _____

c. Mother's Name: _____

2. Age: _____years **3. Gender:** Male/ Female

4. Marital status: Married_____yrs/ Unmarried

5. Area: Rural/ Urban/ Semi urban

6. Address

Permanent:_____

_____

7. Phone: Off: _____ Cell: _____

Res: _____ Tnt: _____

8. Socioeconomic status

- Earning capacity (1. Earner 2. Dependent) _____
- No of earners in the family _____
- No of family members sharing the same kitchen_____
- Totally family monthly expenditure _____

9. Educational status:

☐	Illiterate	☐	SSC or equivalent
☐	Can read only	☐	HSC or equivalent
☐	Can write a letter	☐	Graduate or higher
☐	Other		

10. Impression about social class

☐	Rich	☐	Lower middle
☐	Upper middle	☐	Poor

11. Occupational status

☐	Student	☐	Unemployed
☐	Professional	☐	Housewife
☐	Business	☐	Skilled worker

☐ Technical ☐ Other

12. Religion: Muslim/Hindu/Christian/ Buddhist

13. Any history of past illness or any chronic diseases

a. T2DM Patient history: Hypertension_____ Diabetes_____

Renal disease_____ Cardiac disease_____

Hepatic disease_____

GDM in previous pregnancy_____

No. of child: _____Before 28 weeks of gestation

After 28 weeks of gestation

b. Family history: Hypertension_____ Diabetes_____

Renal disease_____ Cardiac disease_____

Hepatic disease_____

c. Drug History

Trade Name	Generic Name	mg	dose	Duration

14. Physical Activity Level (minutes per day)

Sedentary (Office, teaching, unemployed, walk <30 minutes**:**

Light (Sales, sewing, house work, walk 31-60 minutes :

Moderate (Factory, workshop, walk 61-120 minutes :

Heavy (Laburer, carpenter, rickshaw puller, walk < 121 min:

15. History of addiction

a. Smoking: Yes/ No (if yes; duration _____ yrs; Average ____ sticks/ day)

Past history: Yes/ No (if yes; duration _____yrs; Average ____ sticks/ day)

b. Tobacco leaf chewing/ using powder (Gull): Yes/ No (if yes; duration _____ yrs)

Past history: Yes/ No (if yes; duration _____ yrs)

c. Tobacco Powder sniffing: Yes/ No (if yes; duration _____ yrs)

Past history: Yes/ No (if yes; duration _____ yrs)

d. Drinking alcohol: Yes/ No (if yes; duration _____ yrs)

Past history: Yes/ No (if yes; duration _____ yrs)

16. History of medication

a. Present medications:

b. Past medications:

17. Physical examinations

Anthropometrical measurements:

Ht (m)		Wt (Kg)	
Waist (cm)		Hip (cm)	
Body Fat %		Body Fat Mass	
BP			

18. Previous Biochemical Investigations:

FBS		AG	
TG		LDL-C	
Total Cholesterol:		HDL-C	
S GPT/ALT		S Creatinine	
HbA1C		Insulin	

19. Medical history:

Previous history of hypersensitivity effect to any of the Macroloid:	Yes	No
Taking any medication within 30 days prior to experiment	Yes	No
Abnormalities of menstrual cycle	Yes	No
Previous history of renal dysfunction	Yes	No
Previous history of hepatic dysfunction	Yes	No
Any kind of GIT problem:	Yes	No

20. Food Habit (24 hours recall method)

Morning	
Lunch	
Afternoon	
Dinner	

……

Interviewer Signature