Association of TNF-α, IL6 and IL-10 gene Polymorphisms with Type 2 Diabetes Mellitus in Relation to Insulin Secretion and Action in Bangladeshi Population

Ph.D THESIS

A Thesis Submitted to the University of Dhaka in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in 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 TNF-α, IL6 and IL-10 gene Polymorphisms with Type 2 Diabetes Mellitus in Relation to Insulin Secretion and Action in Bangladeshi Population"** describes here is entirely own of Roksana Yeasmin. 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 has been recommended for the award of Ph.D of Science. To the best of my knowledge, no part of the research work has been submitted for other degrees or qualifications in an institute at home or abroad.

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Dedicated to-

My Beloved Family

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Roksana Yeasmin

Declaration

I hereby humbly declare that this thesis titled **"Association of TNF-α, IL6 and IL-10 gene Polymorphisms with Type 2 Diabetes Mellitus in Relation to Insulin Secretion and Action in Bangladeshi Population"** is based on my own work and has been carried out under the direct supervision of Prof. Yearul Kabir, Professor, Department of Biochemistry and Molecular Biology, University of Dhaka, as a partial fulfillment of the requirement for the degree of Doctors of Philosophy (PhD).

No part of this thesis work has been submitted for another qualification of the University of Dhaka or any other University or any other institute of learning.

Roksana Yeasmin

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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
ADCY5	Adenylate cyclase 5
ADPKD	Autosomal Dominant Polycystic Kidney Disease
ALT	Alanine amino transferase
ANOVA	Analysis of variance
Aps	Action potentials
ATP	Adenosine triphosphate.
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes Endocrine and Metabolic Disorders
BMI	Body mass index
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CE	Cholesterol esters
CETP	Cholesteryl ester transfer protein
CVA	Cerebrovascular accidents
DIAGRAM	Diabetes Genetics Replication And Meta-analysis
sdLDL	Small, dense LDL
DZ	Dizygotic
DBP	Diastolic blood pressure
EDTA	Ethylene diamine tetra acetic acid
ER	Endoplasmic reticulum
FFA	Free fatty acid
FTO	Fat mass and obesity associated
GCK	Glucokinase
GCKR	Glucokinase regulator
GDM	Gestational diabetes mellitus
GINR	Glucose insulin Ratio
GOD-PAP	Glucose oxidase- Peroxidase
GWAS	Genome was association study
HbA_{1c}	Glycosylated Heamoglobin
HDL	High Density Lipoprotein
HDLn	Nascent HDL
HLA	Human leukocyte antigen
HOMAB%	Beta cell function
HOMA-IR	Insulin Resistance
HOMAS%	Insulin Sensitivity

HPLC	High Performance Liquid Chromatographic
HSL	Hormone- sensitive lipase
IDDM	Insulin-dependent diabetes mellitus
IDF	International Diabetes Federation
IGF2BP2	IGF-2 mRNA binding protein 2
IGT	Impaired glucose tolerance
IL-10	Interleukin ten
IL-6	Interleukin six
IR	Insulin resistance
LD	Linkage disequillibrium
LDC	Least developed country
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
MAF	Minor allele frequency
MAPK	Macrophage activating factor
MCP1	Macrophage chemoattractant protein
MNT	Medical Nutrition Therapy
MODY	Maturity-onset diabetes of the young
NCD	Non communicable disease
NIDDM	Non-insulin-dependent diabetes Mellitus
OAD	Oral anti diabetic
PCR	Polymerase chain reaction
PNDM	Permanent neonatal diabetes mellitus
PPAR	The peroxisomal proliferative activated receptor
PPARG	Peroxisome proliferator-activated receptor γ
RE	Restriction enzyme
RFLP	Restriction fragment length polymorphism
RRP	Readily releasable pool
SBP	Systolic blood pressure
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SGs	Secretory granules
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
T1DM	Type 1 diabetes Mellitus
T2DM	Type 2 diabetes Mellitus
TAG	Triacyl-glycerol
TBF	Total body fat
TC	Total cholesterol
TG	Triglyceride
Th1	Helper T cell 1

TNDM	Transient neonatal diabetes mellitus
TNF- α	Tumor necrosis factor-alpha
UTR	Untranslated region
WHO	World Health Organization
WHR	Waist hip ratio

ABSTRACT

Type-2 DM is a polygenic, metabolic illness. It is categorized by prolonged high blood glucose levels as well as unregulated blood pro-inflammatory cytokines by the influence of several genes. A research was conducted on the impact of polymorphism in cytokine genes, with Tumor Necrosis Factor-alpha (TNF-α), Interleukin six (IL—6), and Interleukin ten (IL-10), which consisted of 350 T2DM sufferers and 350 normal healthy controls. The genomic DNA was extracted from the entire blood sample of T2DM affected individuals as well as from control subjects, followed by PCR-RFLP quantification and genotyping using predesigned appropriate primers. The frequency distribution of genotype and allele in T2DM along with controls was examined using SPSS (version 17). Odds ratios (OR) with a 95% confidence interval were established to designate the role of gene polymorphism by the logistic regression analysis. Chi-square tests were analyzed for double and triple combinations of genotypes. Standardized methods were also used to measure the anthropometric (BMI, waist and hip circumferences, waist-hip ratio) and biochemical parameters (FBG, ABF, HBA_1C, fasting insulin, total cholesterol, triacylglycerol, LDL-C, HDL-C). It was found that TNF-α G238A, IL-6 A597G, and IL-10 C592A genotypic frequency followed the Hardy Weinberg Equilibrium in a control population. This study showed non-significant variations in age, sex, Body Mass Index, and the Waist-Hip Ratio between T2DM and control. The only homozygous mutant variant of TNF-α, as well as IL-6 gene polymorphisms, were significantly ($p<0.001$) correlated with T2DM, whereas IL-10 gene polymorphism was significantly ($p<0.001$) correlated with T2DM in both homozygous and heterozygous mutant variant where OR and 95% CI were 3.02 (1.75 - 5.22), 12.28 (7.17- 21.03) and 15.17 (8.98-25.60) and 2.04 (1.43 -2.91) respectively. Minor allele frequency was significantly correlated with T2DM in all three (TNF- α, IL-6, and IL-10) gene polymorphisms. The double and triple combination of the three genes showed a significant association with T2DM. The study outcomes recommend that specific GAA haplotype was significantly ($p<0.05$) correlated with type 2DM. Odds of GAA haplotype had 2.01 times more chances to develop T2DM than the control, and 95% CI was (1.58-2.58). Single nucleotide polymorphism of TNF- α G238A, IL-6 A597G, and IL-10 C592A genes were significantly correlated with T2DM.

1. INTRODUCTION

1.1 Overview

Diabetes mellitus is a group of diseases characterized by high blood glucose levels resulting from insulin production defects, insulin action, or both (Hotamisligil, 2006). The development of diabetes is increasing day by day, and it would reach pandemic proportions over the next 10-20 years (Saxena et al., 2009). The number of human beings affected with diabetes will reach about 642 million by the year 2040 –among which 90% will haveT2DM, and People living in low and middle-income countries will be affected most (Islam et al., 2014). In 2011 the International Diabetes Federation (IDF) estimated that 8.4 million individuals had diabetes, which caused Bangladesh to be 8th in position. Moreover, it remains suspected to be 16.8 million by the year 2030, obtaining the 5th position in rank (IDF, 2011).

Genetic conditions, e.g., monogenic and polygenic mutations and lifestyle factors, as for example, excessive heaviness as well as decreased body exercise and their complex interaction, can result in diabetes mellitus. Relative insulin deficiency or decreased biological function of insulin causes T2DM. Type -2 Diabetes Mellitus (T2DM) or non–insulin-dependent diabetes mellitus (NIDDM) is accounted for 90-95% of diabetic diseases universally. Moreover, it is one of the most typical types of Diabetes. This important disease is produced by either shortage in insulin secretion in the pancreatic beta cells or insulin inactivity in the body cells (Lyssenko and Laakso, 2013). It occurs at the age of 40 (Sarje et al., 2013). Therefore, T2DM is a global problem that desires larger importance on its stoppage, and beneficial policies should be taken in the health care delivery system (Hales et al.,1992). Diabetes mellitus is caused many complications, such as macrovascular and microvascular abnormalities (Maejima et al., 2001).

Moreover, this is well established that T2DM is a polygenic group of disorders resulting from insulin resistance, β-cell dysfunctions, or both. The molecular inherited basis of T2DM was eventually recognized in 2012 (Bajaj and Khan, 2012). That consequently, genetic mutations are caused by diabetes primarily through their effects on β-cell disorder or insulin resistance. The medical characteristics of patients with T2DM are now known to be polygenic, which occurs due to genetic causes. The various genetic makeup of T2DM vary in terms of age of onset, high

blood glucose level, the pattern of diabetes treated by drugs, and whole-body manifestations except for the sign, symptoms of pancreatic diseases. Figure 1.1 depicts that T2DM occurred due to genetic factors and different types of environmental factors such as smoking status, exercise, area of residence, food habits, family history of diabetes, etc. (Scott et al., 2013).

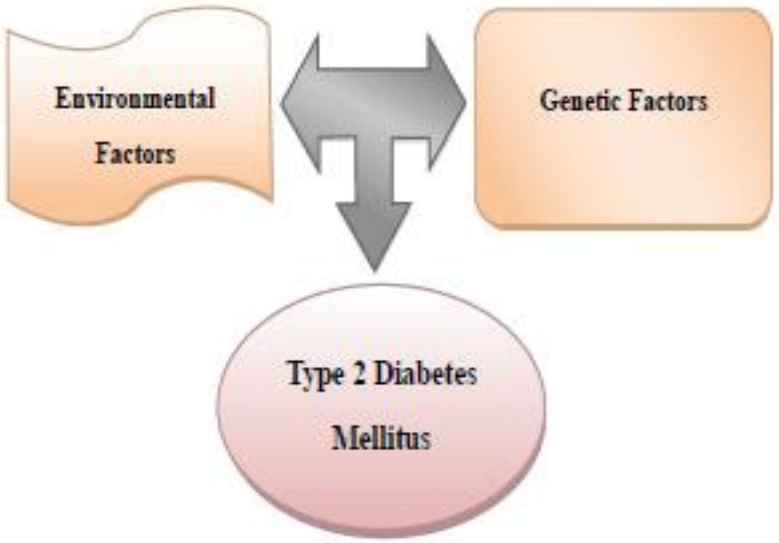

Figure 1.1 Factors Involved in Development of T2DM

Due to the disease's growing burden in diabetic patients, it is important to identify T2DM predictors for managing this disease. Genetic susceptibility is an obligatory factor for diabetes mellitus. Genetic conditions are responsible for about 40% of the factors that cause type 2 diabetes. To see how allelic variance in identified candidate genes related to glucose or lipid metabolism affects type 2 diabetes, researchers looked at a genetic polymorphism in candidate genes. Genetic mutations that affect over 1 percent of an inhabitant's cluster are called gene alternates or polymorphisms. The evolution of T2DM initiates from crosstalk between the genetic material of subjects and their environmental setting. It is correlated with the increasing occurrence of overweightness (Heart et al., 1998), which shows diabetes is spreading in bulk quantity (King et al., 1998). The growth of overweightness seems to be an important feature in signifying insulin resistance progression (Olefsky et al., 2010), which is due to the hereditary setup of beta-cell destruction, which consequences in changes in glucose acceptance.

Inappropriately, although it had earlier been supposed that T2DM was basically a disease of the adult and geriatric population and developed gradually, this hypothesis is currently undergoing re-evaluation. The appearance of T2DM is a new-fangled as well as a severe robustness problem among young adolescents (Rosenbloom et al., 1999; Sinha et al., 2002).

1.2 What is Diabetes?

Diabetes mellitus is a term used to describe a cluster of metabolic disorders caused by faults in insulin activity, secretion, or both. Diabetes mellitus is characterized by long-lasting high blood glucose level and defects in carbohydrate, fat, and protein metabolism as a result of defects in insulin action, secretion, or both (WHO, 2014).

1.2.1 Glucose Metabolism and Diabetes Mellitus

Figure 1.2 shows the overview of glucose metabolism and Type 2 Diabetes Mellitus. After digestion body breaks down the carbohydrates (e.g., sugars and starches) into a simple sugar that can enter the blood directly; throughout the body, cells can absorb glucose using the hormone insulin for energy production (Figure 1.2). Diabetes results from the lack of insulin produced by the body or cannot use insulin successfully or together (Figure 1.2). However, Figure 1.3 depicts that insulin is produced in the beta cell of the pancreas. The pancreas comprises collections of special types of cells named islets. Pancreatic Beta cells in the islets of langerhans produce a hormone named insulin and secrete it into the circulation. If the pancreatic beta cells cannot make an ample amount of active insulin, then serum glucose increases up in the blood (Figure 1.3). Therefore, the blood glucose level increases, which causes metabolic disorders like pre-diabetes or diabetic disorder. However, Pre-diabetes remains a sickness where blood sugars are elevated than ideal but below than to be detected of diabetes. However, Figure 1.4 depicts that higher circulatory sugar can destroy neuron and circulatory blood vessels, causing cardiovascular heart diseases, kidney diseases, loss of sight, and alveolar diseases (Dallas, 2011). Moreover, it causes other problems that may contain amplified vulnerability to additional sicknesses, like mental disorders and pregnancy-related complications. The exact etiology of diabetes mellitus is still not clear. However, researchers trust that genetic and ecological factors are accountable for growing diabetes (Busik et al., 2009).

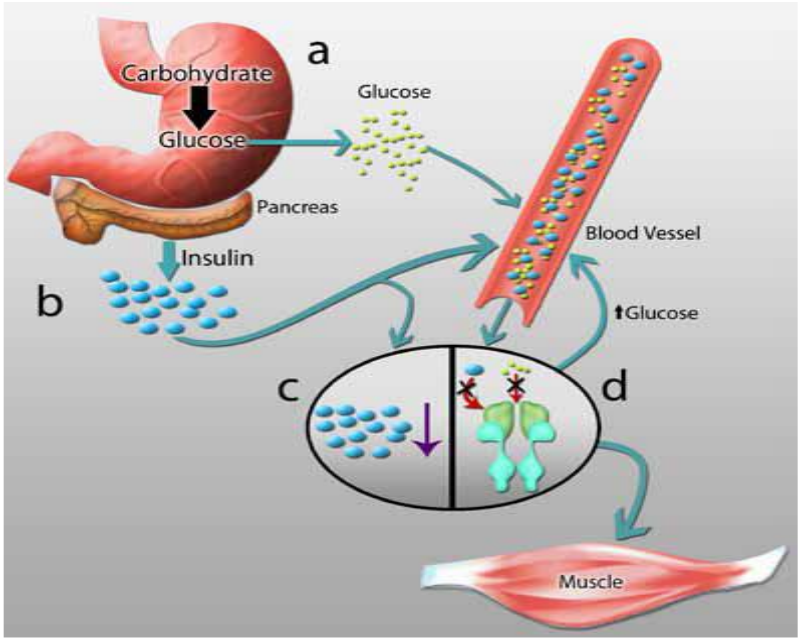

Figure 1.2 Overview of Glucose Metabolism and Type 2 Diabetes Mellitus

a) Carbohydrates, metabolized to glucose in the digestive tract, are released into the 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 decrease in uptake of glucose by organs likes muscles. This ultimately results in increased blood glucose levels and the development of T2DM.

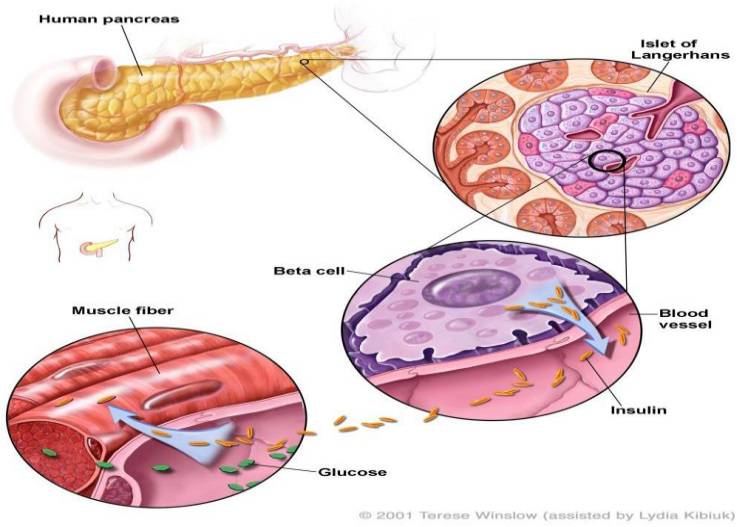

Figure1.3: Pancreas Insulin Production.

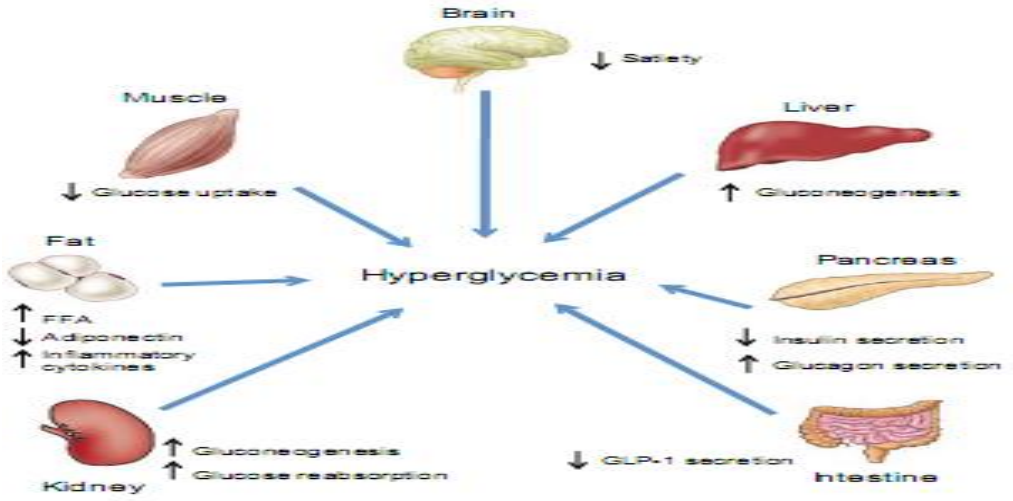

Figure1.4: Multi-organ and Tissue Pathophysiology of T2DM.

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

1.3 Types of Diabetes Mellitus

Researchers have classified diabetes mellitus into two main groups; one of which is insulin-dependent diabetes, which is type 1diabetes, as well as another one is non-insulin-dependent diabetes mellitus that is type 2 diabetes. However, Type 1 diabetes mellitus (T1DM) is distinguished by the self-demolition of pancreatic Beta-cells, which causes successive insulin deficiency. Moreover, T2DM is due to insulin insensitivity and insulin resistance. In some cases, particular mutations cause genetic susceptibilities such as inherited defects of the pancreatic beta-cell activity or insulin function abnormalities. Moreover, another kind of diabetic disease is called gestational diabetes, which occurs only during pregnancy (De Marinis et al., 2010).

1.3.1 Type 1 Diabetes (T1DM)

T1DM is a self-destructive or autoimmune illness. Nevertheless, in the circumstance of self-destructive diseases, where the body's own protective system demolishes its own tissue material (Tao et al., 2015). Usually, youth and teenagers are affected by T1DM diabetic disorder (Busik et al., 2009). T1DM is also recognized as juvenile diabetes (Supply and Programme, 2014). It may be identified as insulin-dependent diabetes mellitus (IDDM) (Guyton and Hall, 2006).

1.3.2 Causes of Type-1 Diabetes

I. Genetic Susceptibility

Many genetic factors interplay among genes impact vulnerability as well as a defense by type-1 diabetes. Moreover, the important genetic factor may differ in changed populations and racial clusters. The risk of type-1 diabetes is related to variants of human leukocyte antigens (HLAs) genes. The production of protein by HLA genes help the immune system acknowledge a cell as a foreign body. Some components of HLA genetic factor variants expect that a person will be at a greater danger for evolving T1DM. Many dangerous genes or gene components have correlated with a remarkable possibility for developing type-1 diabetes. These genetic factors support determining individuals at higher chance for developing type 1 diabetes as well as help to better recognize disease development, identification of probable targets for treatment, and eventually cause the stoppage of the disease (Cornelis et al., 2015).

II. Beta Cells Destruction by an Autoimmune Process

The pancreatic beta cells are destroyed by white blood cells in T1DM. In this case, a person requires a daily dosage of insulin injection to survive. Recent research suggests that insulin might be the prime activator of the body's self-resistance of pancreatic beta cells (Grant et al., 2011).

III. Viruses and Infections

Viruses, for example, coxsackievirus B, cytomegalovirus, adenovirus, rubella, and mumps congenital rubella syndrome, are related to the patient's anti-islet antibodies, which may be connected with type-1 diabetes (Blair, 2016).

IV. Infant Feeding Practices

Dietary factors may increase or decrease the development of juvenile diabetes. As such, breastfed newborns and babies receiving vitamin D supplements are at lower risk to developed type-1 diabetes. At the same time, early introduction to dairy products as well as other proteins derived from cereal may raise the risk of developing T1DM (Tolan and Grant, 2009).

1.3.3 Type -2 Diabetes Mellitus

T2DM is categorized by high blood glucose levels, which happen due to disturbance of faulty insulin release from the beta cell of the pancreas, insulin resistance in distal tissues, and amplified hepatic sugar release (Guillausseau et al., 2008). Moreover, it is the most usual form of diabetes (accounting for 85-95% of all cases worldwide) and is characterized by a disorder of insulin action and secretion, either of which may be the predominant feature. These individuals (previously known as NIDDM) are frequently resistant to the action of insulin (Guyton and Hall, 2006).

Type-2 diabetes is a very common form of diabetes than type 1; in most cases, the beginning of type-2 diabetes occurs after age 30, frequently between the ages of 50 and 60 years, and the disease progresses progressively. As a result, this condition is often referred to as adult-onset diabetes. However, in recent years, the number of younger people with type-2 diabetes has steadily increased, with some as young as 20 years old. This pattern appears to be primarily due to rising obesity rates, which is the most significant risk factor for type 2 diabetes in both children and adults (Guyton and Hall, 2006).

T2DM is usually related to over weightiness, lack of physical exercise, and unhealthy diets. Moreover, it is developed in adulthood. Important treatment strategies for T2DM include lifestyle changes and weight reduction alone, oral medicines, or even insulin injections (Guyton and Hall, 2006).

1.3.4 Important Causes of T2DM

I. Genetic Vulnerability

Genetic predisposition plays an important role in vulnerability to T2DM. However, having definite genes or amalgamations of genes may induce or reduce an individual's possibility of developing the illness. The high incidence of type 2 diabetes in families and identical twins, as well as large differences in diabetes prevalence by ethnicity, indicate that genes play a role (Loos et al., 2015).

II. Over Weightiness and Sedentary Lifestyle

A sedentary lifestyle and over-weightiness are closely correlated with T2DM. Grade 1 obesity causes the inactivity of insulin. Overweightness is more typical in individuals with type 2

diabetes. It could rise from a disparity between energy intake and physical inactivity. Central over-weightiness, in which an individual has excess belly fat, is only a significant risk for insulin resistance. Moreover, T2DM is often indicated by a higher body mass index (BMI) (Nagao et al., 2015; Brestoff et al., 2015).

III. Insulin Resistance

Research states that insulin resistance is common in people who are overweight or obese, have a lot of abdominal fat, and don't exercise (Monteiro et al., 2015). Moreover, insulin resistance occurs when muscle, fat, and liver cells fail to respond properly to the hormone and driving the functioning pancreas to recompense by creating added insulin. Some causes of insulin resistance are high levels of glucocorticoids, increase growth hormone, GDM, polycystic ovarian disease, autoantibodies to the insulin receptor, hemochromatosis (Motahari-Tabari et al., 2015; Biddinger et al., 2006; Chen et al.,1985).

IV. Faults in Insulin release

T2DM pathogenesis is considered by a change in the structure of pancreatic beta-cells correlated with hyperglycemia and insulin resistance (Taddeo et al., 2014; Jing et al., 2008). Beta-cells try for amplified insulin production to tolerate glycemia in response to insulin resistance. Enlarged islet size and proportion of beta-cells are correlated with increased insulin production (Wajchenberg, 2007). As a result, beta-cells weaken insulin secretion. Intracellular triglyceride accumulation can also lead to beta-cell malfunction (Tang et al., 2009; Fink et al., 1983).

V. Glucagon

Glucagon emission by α-cells of the pancreas plays a vital part in sugar maintenance in the blood (Kong et al., 2014). This hormone has a reverse action on insulin. Reduced serum glucose level induces α-cell excretion (Liu et al., 2012). Moreover, in diabetic conditions, glucagon release is not curbed at an increased glucose level, and the release of the hormone is insufficient at a high glucose level (Wajchenberg, 2007).

VI. Oxidative stress

For reactive oxygen species such as superoxide, hydroperoxyl, nitric oxide causes lipid and protein modifications of the cell membrane (Swami and Banerjee, 2013). These reactive oxygen

species cause elevated serum glucose and FFA levels, which contribute to insulin resistance and development in T2DM (Figure 1.5). The increased production of reactive oxygen species along with reduced antioxidant enzyme activity causes the progress of diabetes. Moreover, recent studies have revealed that antioxidants have anti-diabetic properties (An and He, 2016; Ponugoti et al., 2012).

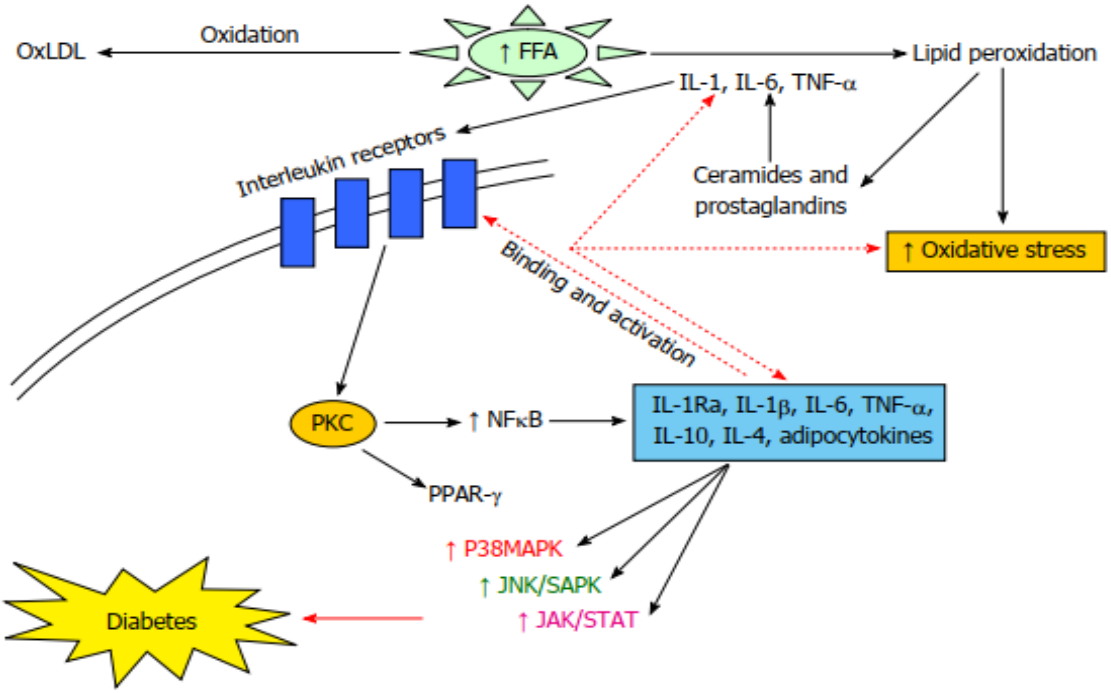

Figure1.5: A Schematic Diagram Showing the Development of Diabetes as a Consequence of Lipid Peroxidation of Cell membrane

Dietary antioxidants counteract oxygen species, which are reactive. Moreover, antioxidants can enhance performance as anti-diabetic drug mediators (Members et al., 2012).

VII. Epigenetic Modifications

Epigenetic alterations modify gene action without changing the whole genome arrangement (Paneni et al., 2013). Environmental factors, improvements of chromatin structures, and transcription machinery also pay to the progression of sugar acceptance, insulin resistance, beta-cell dysfunction, dysfunction of adipose tissue, and T2DM phenotypic expression (Schwenk et al., 2013).

VIII. Polycystic Ovary Syndrome (PCOS)

Polycystic ovary syndrome is connected with obvious upsurges in ovarian androgen creation and insulin resistance. Approximately 6 percent of all women are affected by fatal PCOS at their reproductive age. It is a frequently occurring common endocrine disorder in women (AlFaisal and Al-Deresawi, 2013).

IX. Excess Formation of Glucocorticoids

Excess formation of glucocorticoid hormone (Cushing's syndrome) causes a reduction of the sensitivity of various tissues to insulin's metabolic characteristics. It causes the development of Diabetes mellitus (Hall, 2015).

X. The Liver Produces Glucose in an Unusual Amount

Excess glucose is produced by the liver when glucagon levels increase, contributing to elevated blood glucose levels (Orange, 2017).

XI. Genetic Diseases

Genetic disorders like cystic fibrosis and hemochromatosis are correlated with Type 2 diabetes. Cystic fibrosis, which blocks the pancreas, creates abnormally dense mucus and causes diabetes (Ueno et al., 2009; Oveirne et al., 2018).

XII. Damage to or Removal of the Pancreas

Improper insulin production is correlated with pancreatitis, cancer, and pancreatic trauma. Diabetes occurred as a consequence of the removal of a damaged pancreas and loss of the beta-cell (Fendrich et al., 2009).

XIII. Medications

Insulin action can be impaired due to medicines, for example, nicotinic acid and different types of diuretic medications, medicines acting against seizure, and anti-psychiatric drugs. Pentamidine can raise the chance of pancreatitis, beta-cell impairment, and diabetes. Arsenic has the same thing on the progress of diabetes (Turkoski, 2006).

XIV. Chemical Toxins

According to some researches, a high intake of nitrogen-containing chemicals like nitrates and nitrites can increase the risk of diabetes. Arsenic has also been investigated for potential contributions to the development of diabetes (Wikswo, 2014).

XV. Lipid Breakdown

Lipodystrophy is a result of the reduction or redistribution of fat tissue within the body. Lipodystrophy is correlated with insulin inactivity and type 2 DM (Archer and Blair, 2012).

1.4 Gestational Diabetes (GDM)

The hormone changes and the metabolic demands of pregnancy together cause gestational diabetes. Placenta-produced hormones and other conditions correlated with pregnancy lead to insulin resistance. Heavy or overweight women are at an exceptionally higher risk of developing GDM (Metzger, 2007).

1.5 Risk Factors for T2DM

The most commonly demonstrated risk factors for T2DM are increased body mass index (BMI), hypertension, sedentary lifestyle, poor level of literacy, dietary pattern, presence of diabetes in the family, and even unique genes (Esakoff et al., 2009).

1.5.1 High Body Mass Index (BMI)

Increased BMI is a significant source for the occurrence of T2DM (Ortiz, 2014). Moreover, both males and females have a clear positive correlation between obesity and T2DM (Knowler et al., 1991). The elevated risk of developing insulin inactivity is accountable for type 2 diabetes, that is concerned with obesity (Hebebrand and Hinney, 2009). Over weightiness and insulin resistance genes are affected by ecological aspects such as high-calorie consumption and low physical exercise. This also leads to insulin inactiveness, accompanied by the development of T2DM (Kahn et al., 20012).

1.5.2 Hypertension

Earlier potential and case-control research have shown that hypertensive disorder is a self-governing predictor of T2DM (Kumari et al., 2004). Several possible factors may be responsible for the relationship between T2DM and hypertensive disorder. The endothelial disorder is closely linked to hypertensive disorder (Meigs et al., 2004; Rowe et al., 1983). Finally, insulin resistance could be a probable alternative connection between hypertensive disorder and T2DM (Ferrannini et al., 1987).

1.5.3 Smoking

Smoking is a significant causal factor for the occurrence of T2DM. A retrospective study, including 25 potential types of research, established that smoking is related to about a forty-four percent increase in diabetes-related complications (Talamini et al., 1999). The correlation between cigarette smoking and T2DM was more significant for chain smokers (20 cigarettes/day) than light smokers (Willi et al., 2007; Tao et al., 2015).

1.5.4 Physical Inactivity

A sedentary lifestyle is a vital causal factor for T2DM (Fretts et al., 2009). Watching television for a long time is referred to as a marker of a sedentary lifestyle, and physical inactivity is strongly correlated with diabetes risk in both sexes (Krishnan et al., 2009). Moreover, medium and heavy physical activity reduce the risk of Type-2 DM (Hamman et al., 2006).

1.5.5 Low Education

According to a previous report, a lower level of education was significantly correlated with type 2 diabetes (Maty et al., 2005). Moreover, the National Health Interview analysis found that individuals who drop out of high school have two times more odds of becoming diabetic than those with graduation and post-graduation degrees (Gupta et al., 2015). In addition, the correlation differed on nationality and sex. White and Hispanic women have a higher chance of developing diabetes than black men (Borrell et al., 2006). A new cross-sectional analysis showed that among the least qualified who were obese and inactive, the danger of T2DM was greater relative to the more academic ones (Dashty and Motazacker, 2014).

1.5.6 Dietary Pattern

Nowadays, dietary patterns are a vital lifestyle issue correlated with the expansion of T2DM. A strong constructive correlation demonstrated the risk of T2DM and various food intake cycles. Moreover, an advanced dietetic glycemic index is found to be constantly linked with a greater risk of T2DM in forthcoming cluster research (Schulze et al., 2004). However, probable research shows that constant rice ingesting is connected with the amplified risk of T2DM. In contrast, replacing white rice with brown rice was connected to a lesser risk of T2DM (Hu et al., 2001). Consuming a large amount of green leafy vegetables is correlated with a reduction of T2DM (Schwarz et al., 2009).

The probable proposal recommended that the intake of foods that are rich in fiber is constantly correlated with enhanced insulin activity and declines T2DM (Salmerón et al., 1997).

1.5.7 Genetics

The presence of diabetes in the family among first cousins deliberates an increased risk of T2DM. If both parents are affected, the risk is more significant (Amini and Janghorbani, 2007). Data from several labs confirm genetic factors that predispose to T2DM development by decreasing insulin delicacy and discharge, which in most cases of human T2DM deteriorate in tandem (Ghosh, 2006). The latest studies have identified 11 genetic variants suggestively associated with the risk of T2DM, irrespective of other clinical risk factors, and variants in 8 of these genes are correlated with beta cell dysfunction (Lyssenko et al., 2008; Nagao et al., 2015).

1.6 Relationship between Lipid Profiles and Type 2 Diabetes

Unfavorable blood lipids have been linked to the development of type 2 diabetes. The level of HDL cholesterol and the chance of type 2 diabetes have an inverse relationship. Low HDL cholesterol has been linked to an increased risk of type 2 diabetes in women only, according to some reports. Figure 1.6 depicts that insulin resistance is characterized by high plasma triglycerides and low plasma HDL cholesterol levels. So, insulin resistance is measured by non-fasting triglycerides and HDL cholesterol levels (Tangvarasittichai et al., 2008; Tangvarasittichai, 2015; Dashty et al., 2014; Denou et al., 2015).

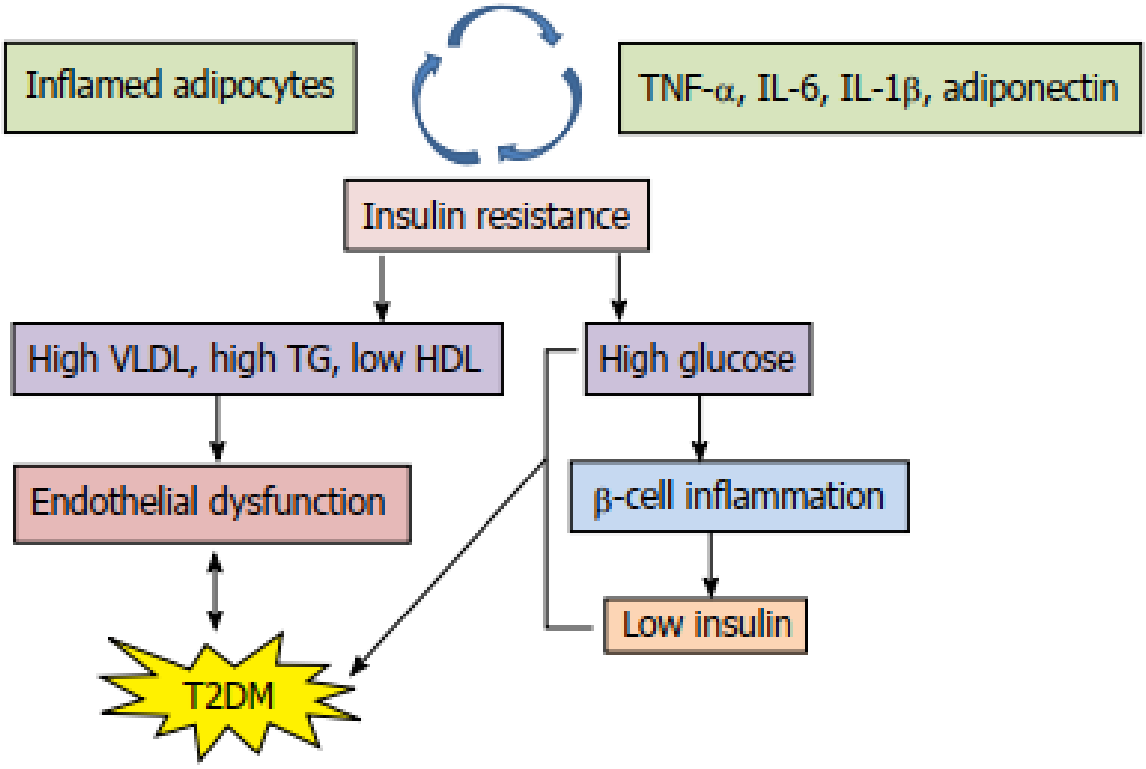

Figure 1.6: A Schematic Diagram Showing the Effects of Lipid Disorder in T2DM

Dyslipidemia, a disorder marked by a low HDL level and a high TG level, is associated with insulin resistance and type 2 diabetes mellitus. Low HDL levels are associated with hyperinsulinemia or insulin resistance. Obesity, metabolic disorder, and T2DM may also show the same dyslipidemia features. On the other hand, TG, HDL, and TG/HDL ratios are self-sufficiently related to serum insulin levels, insulin resistance, and cardiovascular disease risk. In obese, metabolic syndrome, and T2DM patients, both of these characteristics are linked to the risk of coronary heart disease (Tangvarasittichai et al., 2010; Tangvarasittichai, 2015; Li et al., 2011).

As a result, T2DM patients should have their fasting serum lipid profile calculated, as suggested by the American Diabetes Association 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) are all considered low-risk by the American Diabetes Association (Haffner, 2003).

Dyslipidemia is very common in people with type 2 diabetes, with a prevalence of 72–85 percent. In diabetic patients, this effect is linked to a substantially elevated risk of coronary artery disease. Atherosclerosis and other cardiovascular disorders are largely caused by lipid alterations seen in type 2 diabetic patients. These lipid defects are qualitative as well as quantitative and kinetic. Increased triacylglycerols and decreased HDL cholesterol are the key quantitative lipid abnormalities in diabetic dyslipidemia (Turner et al., 1998; parks, 2002; Cabre et al., 1990), which are described in Figure 1.7.

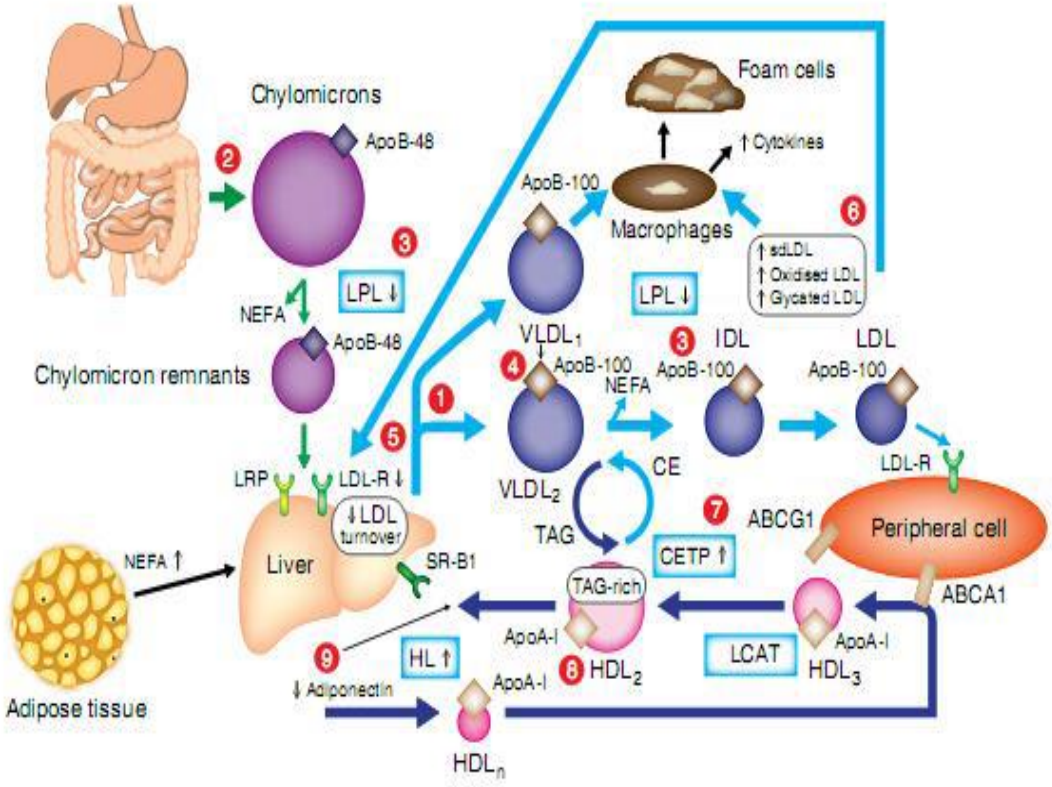

Figure 1.7: Main Lipid Abnormalities in Type 2 Diabetes

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. **Higher Triacylglycerols:** (1) amplified very low-density lipoprotein production (mostly VLDL1), (2) augmented chylomicron creation, (3) amplified creation of very-low-density lipoprotein production (VLDL1), (4) decreased catabolism of both chylomicrons and very-low-density lipoprotein production, favorably occupied up by macrophages.
2. **Higher LDL-cholesterol**: (5) increased number of glycated low-density lipoproteins, small, dense nLDLs (TAG-rich), and oxidised low-density lipoproteins, (6) reduced low-density lipoproteins turnover (decreased LDL B/E receptor), which are favorably occupied up by macrophages.
3. **Low HDL:** (7) increased transfer of triacylglycerols from TAG-rich lipoproteins to low-density lipoprotein and high-density lipoprotein), (8) amplified TAG content of high-density lipoprotein, helping hepatic lipase activity and high-density lipoprotein breakdown, (9) low plasma adiponectin favoring the increase in high-density lipoprotein breakdown (Verges, 2015).

1.7 Procedures for Diagnosing Diabetes Mellitus

At the initial examination, a diabetic condition is diagnosed if any of the following criteria are met: i. fasting plasma glucose level is > 126 mg/dL or >6.0 mmol/L, ii. 75 g OGTT 2-h value is >200 mg/dL or 11.1 mmol/L, iii. Random plasma glucose level is >200 mg/dL or 11.1mmol/L iv. HbA_1c is >6.5 % (Jo et al., 2011).

1.8 Management of Type 2 Diabetes

1.8.1 Assessment of Diabetes

A thorough history of physical examination (including fundoscopy) must be carried out at diagnosis to determine the risk factors and symptoms of diabetes, including Fasting Blood Glucose, Glycated Hemoglobin (HbA_1c), kidney function test, Fat profile, Routine Examination of Urine, Electrocardiogram (ECG). Management should be built on the primary valuation and baseline examinations. Diabetes management involves a lifestyle change, medicine, and self-care, which is encouraged by the patient's education (ADA, 2010). At the time of the initial diagnosis, a thorough examination is needed (ADA, 2010).

1.8.2 Diabetes Education

Diabetes education increases health outcomes and overall quality of life. Diabetes self-care education is known as the method of helping people to control their diabetes, which has been considered an integral aspect of the clinical treatment of people with diabetes since the 1930s. A large body of literature has developed on diabetes education and its efficacy, including several important quantitative reviews showing positive effects of diabetes education (Norris et al., 2002; Phelan et al., 2000).

1.8.3 Lifestyle Modification by Medical Nutrition Therapy (MNT)

A healthy diet is essential for the management of diabetes. To maintain blood sugar and hypertensive disorder, MNT, together with medication, is given to the patient. By balanced eating options, both targets can be accomplished.

A. General Recommendation

- Expert nutritional counseling should be provided.

- Dietary patterns and habits should be tailored following dietary criteria, illness incidence, cultural preferences, and the ability to adapt. (Nisak et al., 2013).
- For overweight or heavy people, a weight reduction of five to ten percent of the actual weight over six months is advised.

This can be achieved by:

- Reduction of calorie intake (twenty-twenty five kcal/kg body weight)
- Increased body fitness by increasing physical activity (150 minutes/week)
- Amendment of behavior
- 50-60% carbohydrate energy, 15-20 percent protein energy, and 25-30 percent fat energy are suggested (Hamman et al., 2006).

B. In Addition to the Above Recommendations

- Meal timings should be regular (avoid skipping meals).

- Daily carbohydrate intake (by carbohydrate exchange) should be monitored as a primary strategy in achieving glycemic control.
- An additional benefit in modulating postprandial response can be attained by using glycemic index (GI) and a load of foods.
- Aspartame and acesulfame potassium are allowed as artificial sweeteners.
- To reduce the risk of CVD, individuals with diabetes must restrict the consumption of saturated fatty acids, trans-fatty acids, and cholesterol (Khan, 1998).

1.8.4 Physical Activity

Improved glycemic control can be achieved by increased physical activity, which can assist with weight maintenance and reduce the risk of CVD.

General Recommendations

- Individuals should exercise 5 days a week, preferably most days of the week, and with no more than two consecutive days without physical activity.
- Brisk walking is recommended for all.
- The duration of exercise should be at least 150 min/week of moderate-intensity aerobic physical activity and/or at least 90 min/week of vigorous aerobic.
- Physical activity should gradually increase to 60-90 minutes per day for overweight and obese individuals for long-term major weight loss.
- Any increase in daily energy expenditure (e.g., gardening, walking, washing the car, mopping the floor) is beneficial (Grigoriev et al., 2009).

1.8.5 Treatment of Diabetes Mellitus

Although it is not possible to cure T2DM completely, it can successfully be treated. The basis for controlling T2DM can be provided by a balanced lifestyle such as diet, exercise, and weight management. However, antidiabetic agents are required to regulate blood glucose levels in serious conditions. There are many factors that influence the treatment of T2DM. Factors include age, physical condition, lifestyle, etc. Insulin can be made more efficient by losing weight and

having more exercise (Kahn et al., 2006; Wajchenberg et al., 2007). Leaving smoking helps to reduce the danger of diabetic complications. Few individuals take medication, whereas other individuals inject insulin or a medicine termed incretinmimetics (hormone-like substances that help the body produce more insulin). Metformin and sulfonylurea are the most effective medicines used to treat T2DM. Numerous people who have T2DM also have elevated blood pressure. The medicines that can reduce the risk of cardiovascular disease are; anti-hypertensive drugs, anti-atherogenic drugs, or reducing cholesterol levels (Association, 2014; Follow et al., 2013).

1.9 Complication of Type 2 Diabetes

1.9.1 Chronic Complication

Cardiovascular diseases, cerebrovascular diseases, nephropathy, ocular lesions, neuropathy, and diabetic foot problems are considered chronic complications that developed for T2DM (Lumeng et al., 2007).

1.9.2 Macrovascular Complication

In type 2 diabetes, macrovascular complications such as coronary artery disease (CAD), peripheral vascular disease (PVD), and cerebrovascular events (CVA) occur. About 50 percent of the deaths in people with T2DM are caused by macrovascular disease (Lorenzo et al., 2007; Giacco et al., 2010).

1.9.3 Microvascular Complication

Microvascular complications comprise retinopathy, nephropathy, and neuropathy (Raciti et al., 2015). Diabetic retinopathy can be well-defined as injury to the small blood vessels in the retina due to long-time hyperglycemia (Billings and Florez, 2010; Gill et al., 2002).

Nephropathy is considered the world's important reason for progressive kidney disease and is accountable for kidney insufficiency. Diabetes causes a group of nerve disorders known as diabetic neuropathies. It is divided into four categories: lateral, autonomic, proximal, and focal. Each has a unique effect on various parts of the body. Diabetic foot ulcers may develop as a result of irregular pressure distribution (Mard-Solta et al., 2012).

1.9.4 Other Complications in Type 2 Diabetes

In people with diabetes, depression is twice as frequent as in general people, and severe depression is present in at least 15 percent of diabetes patients. Another obstacle in diabetic men is erectile dysfunction, and the prevalence was 34 to 45% (Sheikhpour, 2013).

1.10 Prevalence of Diabetes:

1.10.1 The World-Wide Prevalence of Type 2 Diabetes Mellitus

The worldwide number of adults with T2DM at 381.8 million in 2013, The International Diabetes Federation gives a statistic that will grow to 591.9 million in 2035, counting 202 million in the Asia-Pacific region, where diabetes prevalence is also predictable to grow from 8.6% in 2013 to 11.8% by the year 2035 (Aguiree et al., 2013). In the Asia Pacific region, one hundred thirty-eight million (36% of all patients) diabetic patients are type 2 diabetic (Donia et al., 2016). In sub-Saharan Africa, the rise in the incidence of diabetes will be the largest, with a predicted 109.6 percent growth from 19.8 lakhs in 2013 to 41.5 lakhs in 2035 (Table 1.1) (Ting et al., 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 incidence among adults in the United States was five percent in the past nineteen seventies and eight percent in the past nineteen nineties, reflecting a rise of around one point five times over 20 years (Gregg et al., 2004). The prevalence in Japan was 1.6 percent in males and 0.9 percent in females in 1970, slightly inferior to that in the United States of America (Zhang et al., 2011). The occurrence of DM increased by three to five times from about nineteen eighty to around two thousand in other East Asian nations, such as China, South Korea, and Malaysia (Figure 1.8) (Khalid et al., 1998; Islam, 2014; Tilling et al., 2006; Hu, 2001; Kim, 2017).

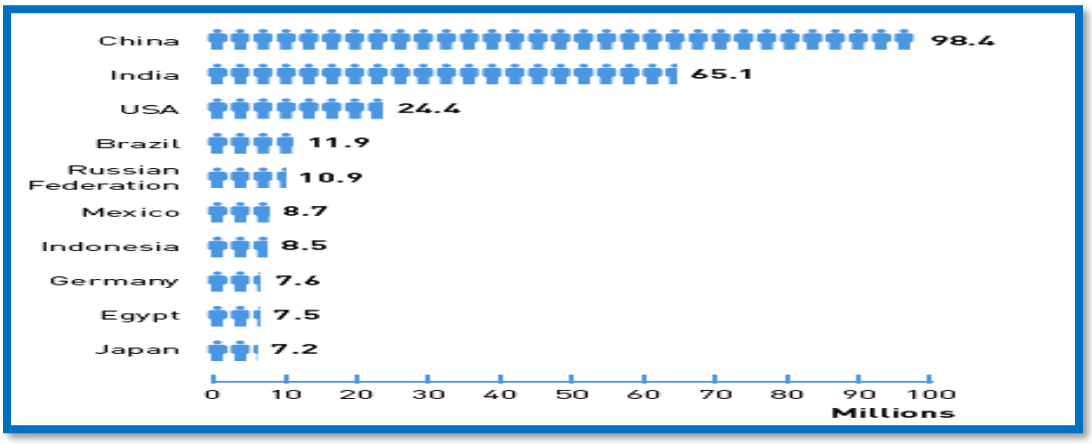

Figure 1.8: Top 10 Countries for Number of People with Diabetes (20-79 years), 2013

The number of people with type 2 diabetes is growing in every single state; eighty percent of people with diabetes live in low- and middle-income states (**Figure 1.9)** (IDF Diabetes Atlas, 2016).

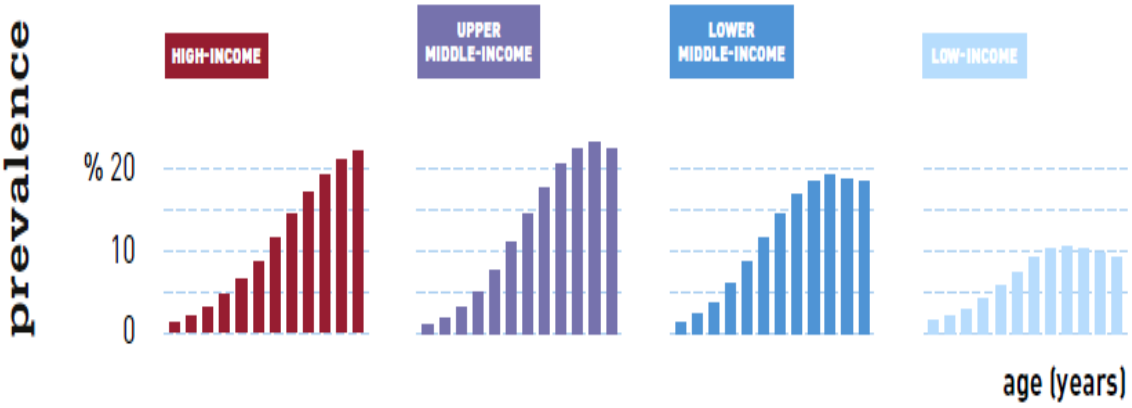

Figure 1.9: Prevalence (%) of Diabetes (20-79 years) by Income Group and Age

Figure 1.10 depicts that the highest numbers of individuals with diabetes are between forty to sixty years of age (IDF 2013). One hundred seventy-five million individuals with diabetes are unidentified in the world. Among them, more than eighty thousand children developed type 1 diabetes in two thousand thirteen, and an additional twenty-one million live births were affected by diabetes during pregnancy in two thousand thirteen. 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 significant area of health-related issues (WHO, 2014).

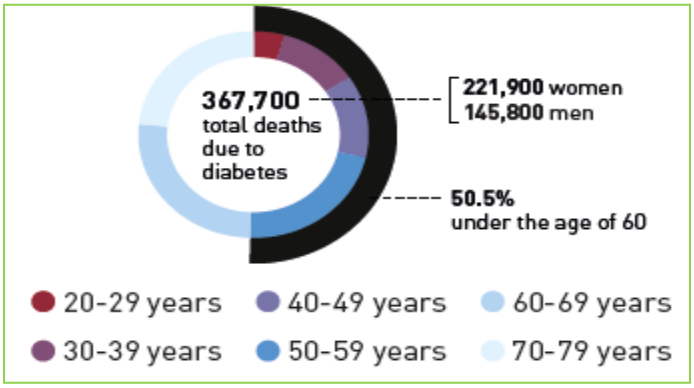

Figure 1.10: Deaths due to Diabetes by Age and Sex

Moreover, this growth is thought to be principally attributed to the ageing of the universal inhabitants, a failure in capability, and rising changes of overweightness. As of 2000, the five nations with the most significant figure of individuals with Diabetes were India, with 31.7 million, China with 20.8 million, the United States of America with 17.7 million, Indonesia with 8.4 million, and Japan with 6.8 million. It is regarded by the World Health Organization as a global disease (Smyth and Heron, 2006; Fasanmade et al., 2008; Larsen and Kronenberg, 2011).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).

1.10.2 Prevalence of T2DM in Bangladesh

The Bangladeshi population has a higher rate of diabetes morbidity and mortality (Saquib et al., 2012). However, the exact reasons or dangerous influences are not recognized for amplified

death in Bangladeshis (Zaman et al., 2016). Moreover, both hereditary predilection and ecological factors are generally accredited regardless of civilization. It places a massive burden not only on human welfare but also on the social and national economy of the household (Shamima et al., 2014). However, from the endocrine records office in Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), it was found that the amount of recorded diabetes in the year 1956 was 39, which has been amplified to 15,296 in 1998. Moreover, among the recorded diabetic affected individuals, 60 percent are male and 62 percent from the city, 32 percent from the countryside, and 6 percent from semi-urban (Akter et al., 2014). 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 (Raja et al., 2014). By the year 2030, the prevalence rate will be 13 percent, according to the International Diabetes Federation (Koh et al., 2013; Ogurtsova et al., 2017)

Bangladesh will be in the top five countries in terms of the number of people living with diabetes in 2030 as a result of this increase in diabetes prevalence. Bangladesh is a least developed country (LDC) with a disproportionately large diabetes rate, according to the United Nations. Bangladesh has more than one-third of the population living with diabetes in the 48 LDCs. Bangladesh is the home to 40% of all diabetics in the world's least developed countries (Novo Nordisk, 2012). The distribution of diabetics in LDCs is depicted in the graph below (Figure 1.11).

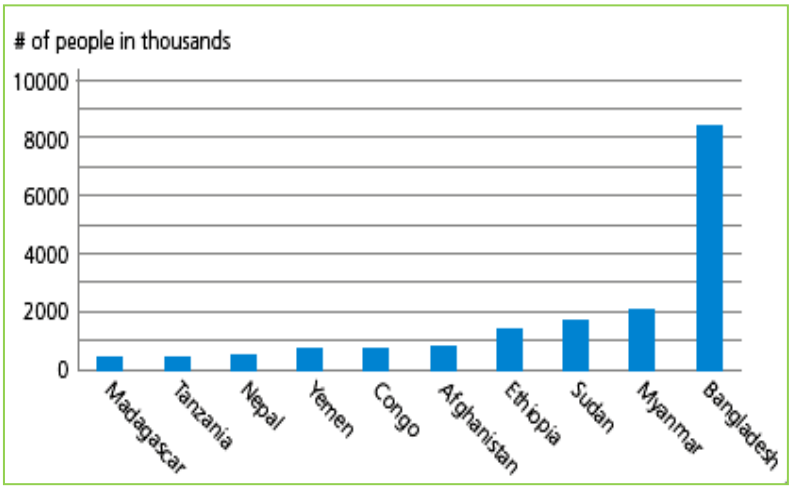

Figure1.11: Distribution of People with Diabetes in the least Developed Countries

A meta-analysis found that the occurrence of diabetes among adults in Bangladesh, which had a population of 149.8 million in 2011, had risen considerably, from 4% in 1995-2000 to 5% in 2001-2005 to 9% in 2006-2010. Diabetes is the tenth most costly condition in Bangladesh in terms of gross healthcare costs for a disorder (Khan et al., 1998; IDF, 2016; Bangladesh population and housing census, 2011). According to studies, the occurrence of type 2 diabetes is between 2% and 21%, and the prevalence of the metabolic syndrome is between 3% and 20% (Zaman and Ahmed, 2006; Jesmin et al., 2012).

1.11 Tumor Necrosis Factor-alpha (TNF-α)

Cytokines are significant intermediates of immunity (Churchill et al., 2006). Tumor necrosis factor- α (TNF-alpha) is a pro-inflammatory cytokine that plays a critical role in multiple autoimmune disorders such as osteoarthritic disorder, hemopoietic disorder, and diabetes mellitus (Werth et al., 2000; Arner, 2000). Fig 1.12 depicts that proinflammatory cytokines like tumor necrosis factor-alpha and interleukin six release from adipose tissue. TNF-alpha is a multifunctional cytokine that can control the function of immune cells, apoptosis, and energy homeostasis in cellular and biological processes (Kawai et al., 2011; Zhang et al., 1998; Day et al., 1998).

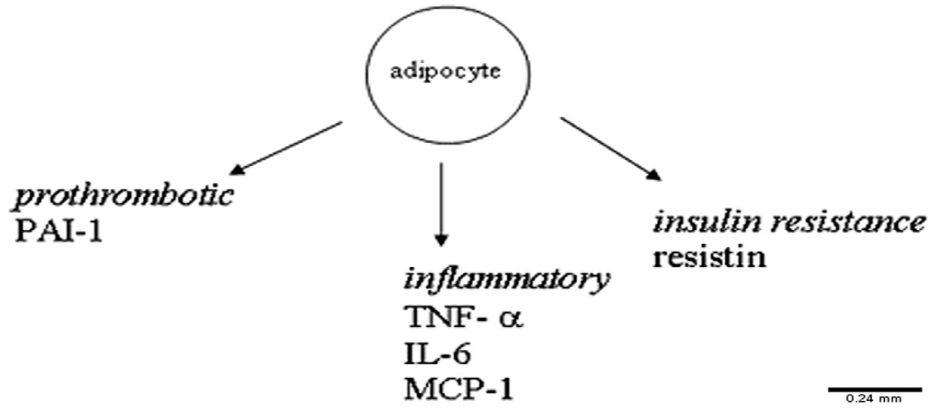

Figure1.12: Cytokines Secreted by Adipocytes.

The locus of the TNF protein is positioned on chromosome 6 (6p21) within the Class three region of the human main histocompatibility complex (MHC) and spans around 3 kb and comprises four exons (Shen et al., 2015). The expression of TNF-α production occurs at both the

transcriptional and post-transcriptional levels (Tsuji et al., 1991). Regulatory sequences are within the 59 ends of the gene controlling the rate of transcription (Behnam-Rassouli et al., 2010). In the human TNF-alpha(α) gene promoter region, many single-nucleotide polymorphisms (SNPs) have been recognized (Adamo and Barone, 1997). The position of the TNF gene on MHC has increased the probability of polymorphisms which can cause the pathogenesis of several autoimmune and infectious diseases (Spriggs et al., 1992). It is developed by monocytes, macrophages, CD4+ and CD8+ T cells, B cells, lymphokine-activated killer (LAK) cells, natural killer (NK) cells, endothelial cells, several non-hematopoietic tumors cell lines (Kubaszek et al., 2003). There are some grounds of TNF-alpha activation, such as mast and neutrophil cell activation (Porta et al., 2007). TNF-α receptors are expressed by an extensive diversity of cells and tissues (Dalziel et al., 2002). This recommends that TNF-α has taken part in a number of biological activities (Vilcek and Lee, 1991; Heng et al., 2008). Gene polymorphism located at position 238 (guanine [G] replaced adenine [A]) in the 5' regulatory region of the gene can increase the expression of this cytokine in fat tissue and affect fat mass and insulin resistance (Popko et al., 2010). Induction of insulin resistance may cause different metabolic syndromes, such as T2DM (Torti et al., 1985). The presence of the A allele induces a double rise of TNF-α gene expression; as a result, polymorphism leads to higher TNF-α production (Brandt et al., 2001). Many studies have found that people who have the 238A TNF-alpha gene mutation are in danger of transitioning from reduced glucose tolerance to type 2 diabetes (Di Rocco et al., 2004) and type 1 diabetes (Simoneay et al., 2014). There were also some studies that found no relation between a mutation in TNF-α and insulin resistance or any other metabolic abnormality (Day, 1998). TNF-α expression in adipocytes is significantly correlated with high body mass index, percentage of central body fat, and hyperinsulinemia; in the case of weight loss, TNF-α level decreases (Cawthorn and Sethi, 2008). Polymorphism at 238 G>A positions of the promoter of the TNF-α gene increased the transcription of the gene in adipose tissue (Jellema et al., 2004); TNF-alpha, messenger RNA levels, are inversely correlated with lipoprotein lipase production in the human muscular system and adipose tissue (Kern et al., 1995).

Two nucleotide polymorphisms found in the promoter region of the TNF-alpha gene have recently been the subject of attention: G >A substitutions at -308 and -238, respectively (Lamzin and Wilson, 1997). Significant associations of these polymorphisms are found in relation to the expression of the gene in the lymphocytes (Hotamisligil, 1999). These TNF-α promoter

polymorphisms play the role as indicators for adjacent genes encoding HLA or other undefined molecules in the MHC that may impact disease vulnerability in humans (Uglialoro et al., 1998).

TNF-alpha is a multifunctional cytokine that can influence multiple biological and cellular processes, such as immune cell activity, apoptosis, and homeostasis of energy (Souza et al., 2003). The position of its gene within the diverse and biological activities of MHC has elevated the likelihood of polymorphisms that can lead to the pathogenesis of an extensive variety of autoimmune and contagious diseases within this locus (Nawashiro et al., 1997; Hotamisligil et al., 1993).

The TNF 238 G>A polymorphism was demonstrated to control the transcription of the gene and analyzed by many researchers in many areas like diabetic nephropathy and retinopathy (Molvarec et al., 2008; Pociot et al., 1993).

Genetic polymorphisms are also analyzed at−862, −856, and −574 relative to the TNF-α transcription start site (Stonek et al., 2008; Di Rocco et al., 2004). Polymorphisms are analyzed in several positions such as; -238, -244, -274, -308, -376, and +70 positions in several studies (Zhang et al., 2011; Legler et al., 2003). The −238, −244, −308, and −376 polymorphisms demonstrate base variations at the particular positions, whereas the +70 polymorphism is an additional nucleotide of the TNF alpha gene (Allen, 1999; Xu et al., 1998; Stonek et al., 2008). Significant associations of these polymorphisms are found concerning the expression of the gene (Enayati et al., 2015; Kaluza et al., 2000).

TNF 238 G>A polymorphism with the exchange of guanine (G) by adenine (A) was found in 1994 and is situated within a putative Y box (Hamaguchi et al., 2000; D'Alfonso and Richiardi, 1994). A TNF-α repressor site was identified between -254 and -230 of the gene (Fong et al., 1994; Hastuti et al., 2017). In one study conducted in UK in 1998, TNF 238 G/A polymorphism was correlated with reduced insulin activity (Day et al., 1998; Kern et al., 1995; Buntinx et al., 2004). However, there were scientists (Sheu et al., 2001; Valenti et al., 2002; Hotamisligil et al., 1996) who did not find any correlation between TNF 238 promoter polymorphism and insulin resistance. Polymorphisms of the TNF-alpha gene and their effects on the level of TNF-alpha in blood and diseases have been studied (Hajeer and Hutchinson, 2001). But the result shows significant differences according to gender, social background, and geographical variation

(Jefferis, 2009; Hawrami et al., 1996; Barone et al., 1997). Several studies have shown the relationship between TNF- α protein and diabetes (Wilson et al., 1993; Goldman and Yang, 1994; Mueller et al., 1995). But, the correlation between this genetic variation and the chance of occurrence of T2DM still remains indistinct (Hussain et al., 1996; Yuuki et al., 2001; Heiermann et al., 2011). Therefore, this gene can be considered a risk factor for T2DM pathogenicity (Dziembowski et al., 2010; Ding et al., 2013; Forouzanfar et al., 2014).

1.11.1 TNF-α and Insulin Resistance

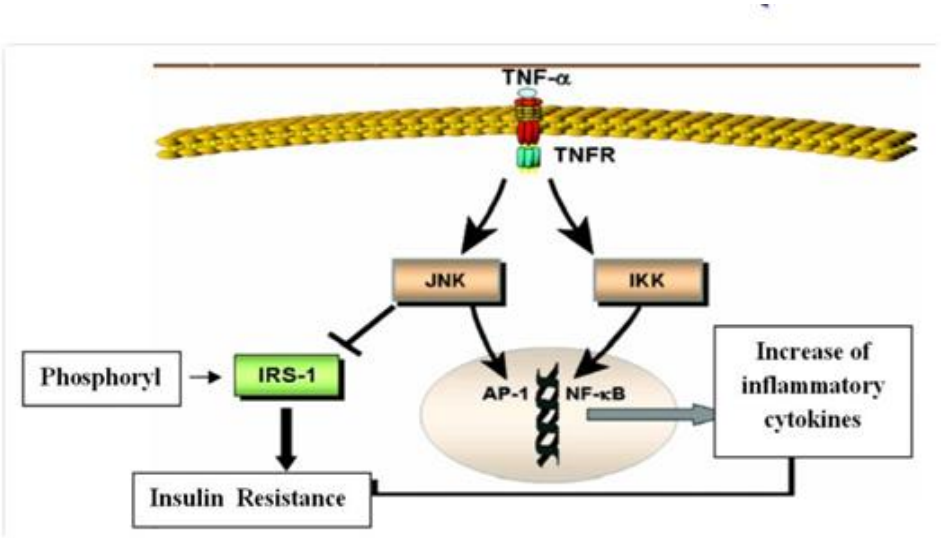

Figure 1.13: Molecular Mechanism of Insulin Resistance by TNF- α

Figure 1.13 shows the molecular mechanism of insulin resistance by TNF- α. The key metabolic abnormality of insulin resistance in type 2 diabetics is a deficiency in glucose absorption caused by a malfunction in the glucose transporter-4 (GLUT-4) protein's control (Figure 1.13). The inhibition of tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) occurs due to the abnormality in translocation of GLUT-4 (Lin et al., 2005). Inhibition of binding and activation of phosphatidylinositol 3-kinase (PI3K) and the initiation of downstream signaling pathways result from serine phosphorylation of IRS-1 (Nelson et al.,2000). Cell sensitivity to pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α) or elevated levels of free fatty acids (FFAs) has been shown to have an inhibitory effect on IRS-1 phosphorylation (Özcan et al.,

2004), inhibiting downstream signaling pathways and the activity of insulin (Kaneto et al., 2005). This is the molecular mechanism of insulin resistance by TNF- alpha.

1.12 Interleukin-6 (IL6)

Interleukins are a wide variety of small cell-signaling protein molecules or cytokines that control the immune system's function in humans (Lippitz, 2013). T cells, monocytes, macrophages, and endothelial cells are primarily formed by interleukins (Kamath et al., 2011). Their functions include promoting immune cell contact, gene regulation, controlling transcription factors, and regulating the secretion of inflammation, differentiation, proliferation, and antibodies (Feuerer et al., 2009). Interleukin genetic polymorphisms act as a pivotal role in their activity and change the function of cytokines and deregulate their expression (Li et al., 2007; Hotamisligil et al., 2006).

Interleukin 6 (IL-6) is both a pro-inflammatory as well as an anti-inflammatory cytokine that functions as an interleukin. The IL6 gene is encoded by the 7p15-p21 (Pal et al., 2014). In order to activate the immune reaction, IL-6 is secreted by T cells and macrophages, e.g., during infection and after wounds, in particular after burns or other tissue injury contributing to inflammation (Cardellini et al., 2005). IL-6 also plays a role in the battle against the disease, as IL-6 has been demonstrated to be necessary for resistance to streptococcus pneumonie bacteria in mice (Kawai and Akira, 2011). IL-6 is also developed by smooth muscle cells in the tunica media of several blood vessels as a pro-inflammatory cytokine (Purohit et al., 1995). The role of IL-6 as an anti-inflammatory cytokine is mediated by its inhibitory activities on TNF-alpha and IL-10. Immune cells, adipose tissue, and muscles secrete IL-6 and can speed up or suppress inflammatory processes (Tsigos and Chrousos, 2002). The potential effect of IL-6 may be on glucose homeostasis and metabolism, or it might act on islets of Langerhans (Ruan and Lodish 2003). Insulin tolerance was observed to correlate dramatically and to make it an attractive candidate gene with elevated plasma IL-6 levels with a severe risk of T2DM (Stephens et al., 1992). One of the typical polymorphisms (IL6-174 G/C) was analyzed to control transcription in relation to inflammatory stimuli, such as lipopolysaccharides (Kanety et al., 1995). In 1991, research was conducted on the correlation between diabetic nephropathy and IL-6. The results of the research concluded that there were increased serum levels of IL-6 in patients with type 2

diabetic nephropathy than in control (Sheikhpour, 2013). Therefore, the progression of T2DM can be closely related to human IL6 promoter polymorphism (Simmonds et al., 2004).

1.12.1 Molecular mechanism of Insulin resistance by both TNF-α and IL-6

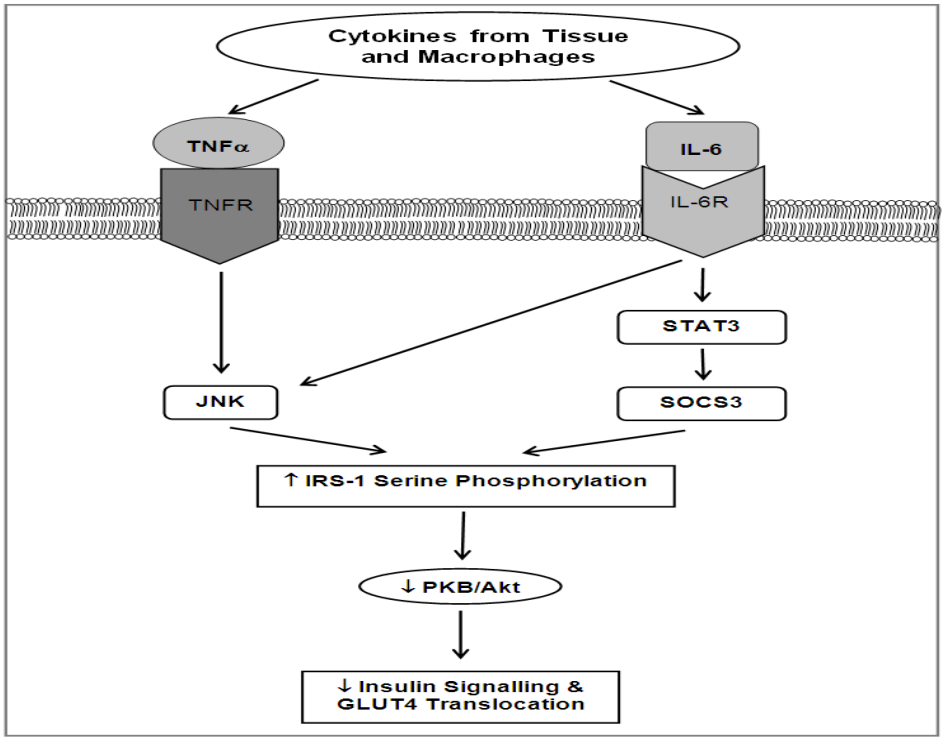

Figure1.14: Molecular Mechanism of Insulin Resistance by both TNF- α and IL-6

Figure 1.14 shows the molecular mechanism of insulin resistance by both tumor necrosis factor-alpha and interleukin six. It shows that IL-6 binds with its receptor and activates the $STAT_3$, $SOCS_3$, which causes serine phosphorylation of IRS-1. This, in turn, blocks the insulin signaling pathway (Kubaszek et al., 2003).

1.13 Interleukin-10 (IL-10)

Interleukin-10 (IL-10**)** is a helper two cells (TH2) mediated cytokine that inhibits the cytokines' provocative actions, helping develop inflammation. It is also an anti-inflammatory cytokine (Moore et al., 2001). It is an immune-regulatory cytokine, which regulates T cells and monocytes or macrophages (Donia et al., 2016; lech-Maranda et al., 2004). Moreover, IL-10 is

abundantly formed by monocytes and TH2, mast cells, regulatory T cells, along with a substantial subset of activated T and B cells (Said et al., 2010). Furthermore, in humans, IL-10 is encoded by the IL-10 gene located on chromosome 1(q31– 1q32) and comprises five exons (Mohebbatikaljahi et al., 2009; Kim et al., 1992). Three specific single nucleotide polymorphisms (SNPs) have been identified (Chang et al., 2005). Several studies demonstrated that there is a significant association between blood level of IL-10 and whole-body insulin sensitivity (Organization, 2003) and have been revealed that reduced IL-10 secretion was correlated with high blood glucose level (Van Exel et al., 2002). Several studies have been recommended that the gene polymorphism of IL-10 has a definite role in defining diabetic occurrence (Straczkowski et al., 2005). Interleukin 10 (IL-10) is diverse against inflection response regulatory cytokine that works as a general proliferative and cytokine response inhibitor of both type 1 and type two helper T cells (Del Prete et al., 1993). Therefore, the IL10 polymorphisms at –1082 G>A (rs1800896), -819T>C (rs1800871), and -592 C>A (rs1800872) improve IL-10 gene transcription (Zhang and Wang, 2006). Moreover, researchers referred to that low IL-10 creation is related to metabolic disorder and T2DM (D'Alfonso et al., 2000; Molofsky et al., 2010). The polymorphism of the IL-10 gene at a specific locus causes a variation of IL-10 creation. Thus, an investigation of genetic variances of IL-10 may describe individual variations in risks of T2DM. Moreover, recent molecular epidemiological research shows that IL-10 C592A gene polymorphisms increase the risk of T2DM (Del Prete et al., 1993; Olefsky et al., 2010).

1.13.1 Molecular Mechanism of Insulin Sensitivity by IL-10

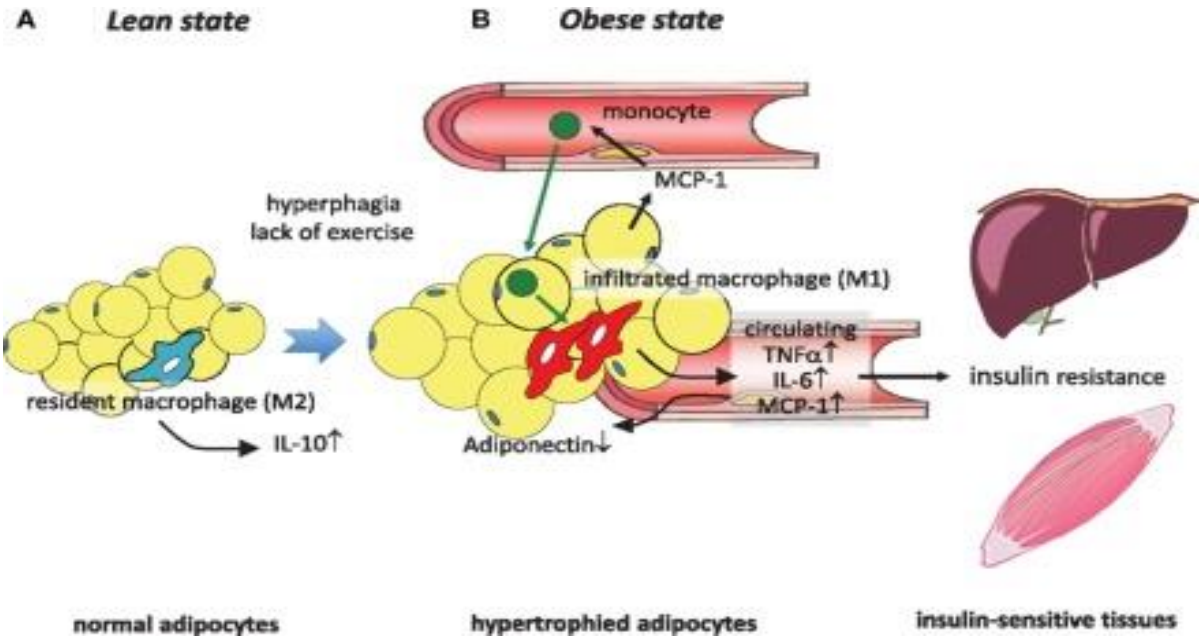

Figure1.15: Molecular Mechanism of Insulin Sensitivity by IL10

Figure (1.15) shows the molecular mechanism of insulin sensitivity by interleukin ten. It shows that in the lean state (A), resident macrophage (M2) of adipocytes releases interleukin ten and increases insulin sensitivity of IL-10, whereas in the obese state (B), infiltrated macrophage (M1) of adipocytes releases proinflammatory cytokine (Saxena et al., 2013).

1.14 Hypothesis

It was hypothesized that Single Nucleotide Polymorphisms of TNF-α, IL-6 and IL-10 genetic variants could be associated with T2DM patients in relation to insulin secretion and action in Bangladeshi population.

1.15 Objective:

1.15.1: General Objectives of the Study:

To estimate the association of Tumor Necrosis Factor-α, Interleukin-6, and Interleukin-10 gene polymorphisms with T2DM in relation to insulin secretion and action in the Bangladeshi population.

1.15.2: Specific Objectives of the Study

1.To find out different polymorphic variants of TNF- α, IL6 and IL10 gene related to type 2 diabetes mellitus.

2.To identify the genotypic distributions of the TNF- α, IL6 and IL10 gene variants in type 2 diabetic and non-diabetic subjects.

3. To rule out the polymorphisms of TNF- α, IL6 and IL10 genes associated with type 2 diabetes among Bangladeshi populations.

4. To see the association of lipid profile with the TNF- α, IL6 and IL10 gene variants in Bangladeshi population

5. To find out the association of glycemic profile with the TNF- α, IL6 and IL10 gene variants in Bangladeshi population.

6. To investigate the association of polymorphisms of TNF- α, IL6 and IL10 gene to the susceptibility and severity of type 2 diabetes mellitus.

1.16 Significances of This Study

This study will help us to evaluate the correlation of Tumor Necrosis Factor- α, IL6, and IL10 gene variation with T2DM in Bangladeshi people. To the extent of my knowledge, there were no studies conducted on the role of TNF-α, IL6, and IL10 genetic variants in the incidence of T2DM in the Bangladeshi population. However, the result of many types of research inclined to the fact that the Bangladeshi community is more susceptible to the growth of T2DM problems. Increased prevalence possesses a severe problem in the healthcare system of Bangladesh. If the gene polymorphism responsible for increased susceptibility to T2DM is determined, then protective and preventive measures can be taken. If we can conduct multiple researches on T2DM carrying polymorphic variants of TNF alpha, IL6, and IL10 gene, then we can prove its

harmful effects and can create awareness among the people to avoid lipid-rich diet in order to increase the blood HDL-C level. The knowledge of gene variants and associated risks will lead to the development of personalized medicine and nutrition against mutant variants of TNF alpha, IL6, and IL10 genes. Hence, this will cause a reduction in the economic burden of society.

2. Materials and Methods

2.1: Study Period: The research was performed from September 2015 to December 2020

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

(i) Case: 350 T2DM patients

(ii) Control: 350 subjects without a history of T2DM

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

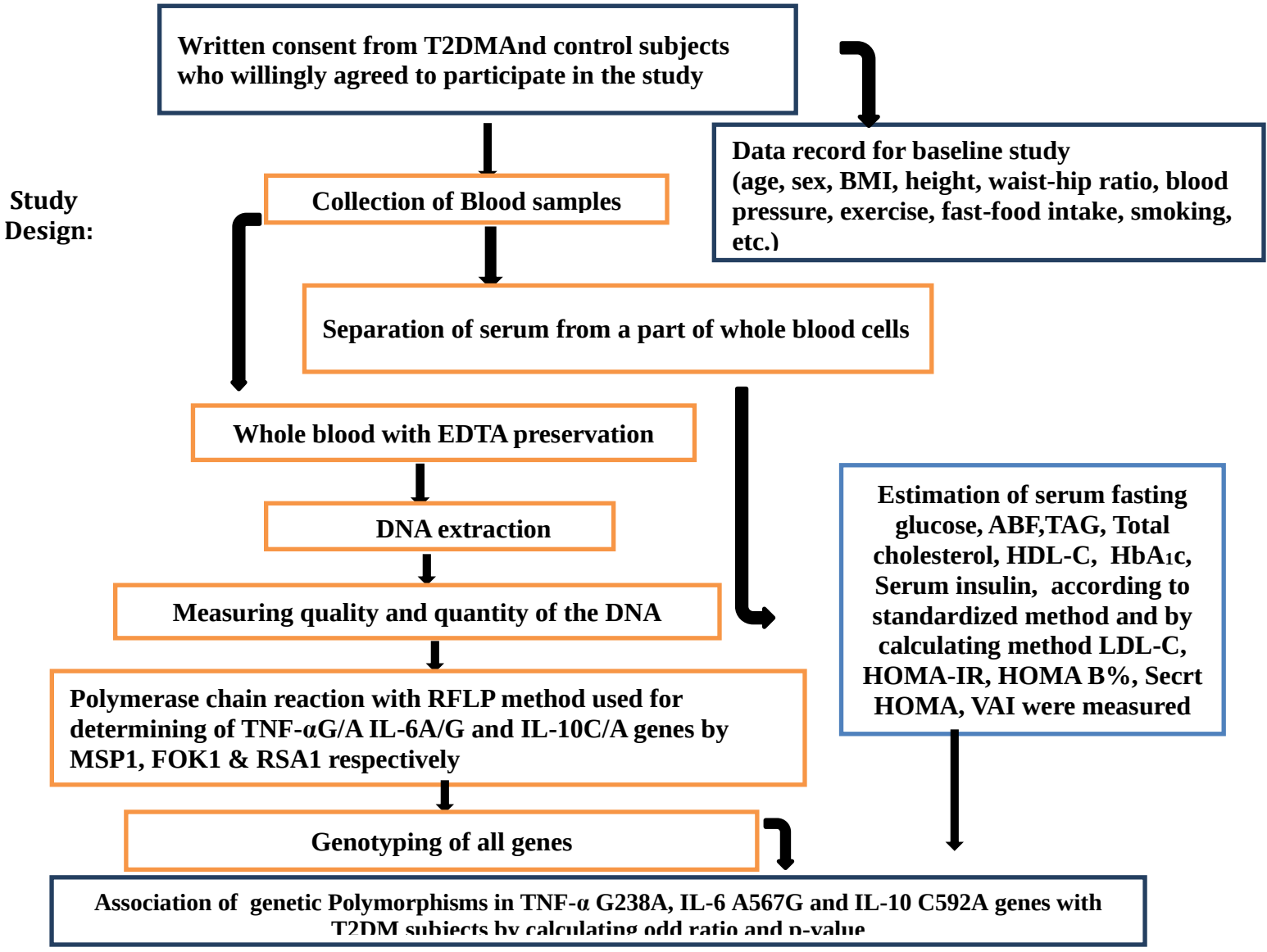

Figure 2.1: Experimental Design of the Study

2.2.1 Subjects of the Study

The research was conducted on 350 type 2 diabetic patients and 350 normal healthy controls of both genders. These T2DM patients were selected from the outpatient departments of BIRDEM. The type 2 diabetic patients were defined based on fasting blood glucose (FBSG) and 75 g oral glucose tolerance test (OGTT). Three hundred and fifty normal healthy participants with a negative history of diabetes or any other chronic illness were recruited as control. Controls were selected from workers of BIRDEM and employees of the residential hall campus of Dhaka University. The type 2 diabetic subjects were matched by age and sex with the control subjects. The sociodemographic, clinical, and biochemical data, including gender, age, area of residence, systolic blood pressure (SBP), diastolic blood pressure (DBP), body mass index (BMI), visceral adiposity index (VAI), waist and hip ratio (WHR), fasting blood glucose (FBG),2 hours after breakfast blood glucose (ABF), HBA_1C, and duration of diabetes, exercise history, hypertension, drug history, smoking history and fast food taking history/week were collected from the people who participated in the study, during the time of whole blood collection. To identify type 2 diabetic patients, "The American Diabetes Association Guidelines" were strictly followed. The guideline is given below:

Plasma glucose levels for diabetic and control groups have been selected according to table 2.1.

Table 2.1:Criteria of Fasting Plasma Glucose Levels and 75g Oral Glucose Tolerance Test after 2-hours Value.

	Normal range	**Diabetic range**
Fasting value	**<110 mg/dl (<6.0 mmol/L)**	**126 mg/dl (7.0 mmol/L)**
75 g OGTT 2-h value	**<140 mg/dl (<7.8 mmol/L)**	**200 mg/dl (11.1mmol/L)**

Source: (American diabetes association, 2014)

2.2.2 Inclusion Criteria of Case

- T2DMsubjects
- Duration of diabetes: 2-10 years
- Age 30-60 years.

2.2.3 Inclusion Criteria of Control

- Non-diabetic healthy volunteers
- Age 30-60 years

2.2.4 Exclusion Criteria of Both Case and Control

- Evidence of any kind of acute infection and any other systemic disorder
- Evidence of hepatic dysfunction: ALT (SGPT) or AST (SGOT) >100 units
- Evidence of renal dysfunction: S creatinine> 1.7mg/dl
- Presence of malabsorption syndrome
- Presence of autoimmune disease
- Pregnant women
- Recent history of cardiovascular diseases
- cancer

2.3Ethical Issue and Consent

The thesis was approved by the Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) and the Department of Biochemistry and Molecular Biology, University of Dhaka, and it was carried out in accordance with the Helsinki Declaration. 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 use only for research purposes. After signing a "Consent Form," the participants were encompassed in this research, and blood was taken.

2.3.1 Questionnaire

The patient's visit was scheduled prior to the beginning of the study. After the collection of blood, baseline characteristics, including anthropometry and clinical examinations, were done. The questionnaire covered all study subjects' information on age, gender, medical, educational, drug history, exercise history, fast food taking habits, history of hypertension, and history of other chronic diseases were taken.

2.4 Collecting and Storing of Blood Samples

Blood samples from the T2DM patients and control subjects were collected in 2 conditions, one while fasting and another exactly 2 hours after breakfast. About 8 (eight) mL of venous blood was drawn from each participant by a technician, ensuring all aseptic precautions using a 10 mL disposable syringe in fasting condition, and 2 hours after breakfast, 2 mL venous blood was drawn from each participant in another 3 mL disposable syringe. Approximately four (4) mL blood was immediately transferred to a vacutainer tube for estimation of fasting glucose, lipid profiles, and fasting insulin, and four (4) mL blood was preserved with EDTA-Na2 containing tubes separately for HbA1C estimation and DNA extraction, respectively; and kept in an ice box for transportation to the laboratory. Serum samples were separated from vacutainer tubes after centrifugation for 10 minutes at 3,000 rpm. Samples for HbA1c was estimated within 1 hour, and other samples (serum and whole blood) were stored at -200 and -800C until further use, respectively.

2.5 DNA Extraction and Quantification

2.5.1 Extraction of DNA

DNA was separated from whole blood through the method described by Hosen (Hosen et al., 2015).

2.5.1.1 Chemicals and Reagents for DNA extraction

1. EDTA (0.5 M), pH 8.0

18.61 g of anhydrous EDTA (Merk) was added to 80.0 mL of distilled water. The pH was adjusted to 8.0 with NaOH (Merk) pellets using pH meter. The solution was made up to 100 mL with distilled water. It was autoclaved at 15 p.s.i. for 15 min.

2. Tris-HC1 (1 M), pH 7.6

12.11 g of Tris base (LOBA Chemie) was dissolved in 80.0 mL of distilled water. The pH was adjusted with concentrated HCl (Merck) by pH meter (Hanna instrument, pH 211). The solution was cooled at room temperature before the adjustment of the pH. The volume was made up to 100 mL with distilled water and autoclaved at 15 p.s.i. for 15 min.

3. Red Blood Cell Lysis Buffer

1.0 mL of Tris (1 M), 10.954 g of sucrose (LOBA chemie), 0.101 g MgC12 (Merck), and finally 1.0 mL of Triton X-100 (Roth) were taken into 80 mL of distilled water, and the pH was adjusted at 8.0. Then the volume of the solution was made up to 100 mL with distilled water and autoclaved at 15 p.s.i. for 10 min.

4. Nucleic Lysis Buffer

1.0 mL of Tris-HC1 (1 M, pH 7.6), 0.375 g of anhydrous EDTA (pH 8.0), 1.0 g of SDS (Merck), 0.294 g of sodium citrate was taken in a volumetric flask, and the pH was adjusted to 8.0. The volume was made up to 100 mL with distilled water. It was autoclaved for 15 min at 15 p.s.i.

5. Chloroform (Roth), prechilled to 4 °C

6. 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.

7. Ethanol (Merck) (100%), prechilled to -20 °C.

2.5.2 Extraction Procedure

At first, we took 500 µL of blood and then poured it into a 1.5 ml Eppendorf tube, and 1000 µL of RBC lysis buffer was added. It was shaking gently and then spinning for two to three minutes at 6000 to 7000

rpm using a centrifuge machine. The supernatant was then discarded, and two or three more times, we repeated the above procedure to eliminate Hb. The tube was placed downward on tissue paper for a few seconds. Moreover, we added 400 µL of nucleic lysis buffer, 100 µL of saturated NaCl (5M), and 600 µL of chloroform to the eppendorf tube. Afterward,spinned it for two to three minutes at 6000 to 7000 rpm, and 400 µL of supernatant was moved to a newly taken 1.5 ml eppendorf tube. Moreover, ice-cold (-20 ˚C) absolute ethanol of 800 µLwas added and then shook slowly. The Eppendorf tube was spin for one minute at 12,000 rpm to precipitate the DNA. Then the supernatant was discarded cautiously as well as the tube was totally dried out at an average temperature, placing the bottom of the tube at an upright position on a toilet paper. Finally, 100 µL of distilled water was added to the Eppendorf tube containing DNA, and after dissolving DNA, the tube was kept at -20˚C for later uses.

2.5.3 Quantification and Purification of DNA

The quantity and purity of the genomic DNA were determined by a NanoDrop spectrophotometer. The absorbance of diluted DNA was measured at 260 nm and 280 nm.

2.6 PCR Amplification of the Target Genes Segment

The TNF- α, IL-6 and IL-10 genotypes were determined using the PCR-RFLP method.

Reagents

- Go Taq Polymerase (Promega, 195652)
- dNTPs (Promega, 104487)
- Primers
- DMSO (Merck, k852147963)
- Restriction enzymes (Jena BioScience, IC03214038)
- Agarose (Roth, 073196818)
- Ethidium bromide (Sigma Chemical Co. (U.S.A.)
- 100 bp D.N.A. ladder (GeneON, 304005)

2.6.1 PCR of the TNF-α Gene

A reaction volume of 15 µLwas taken, and a polymerase chain reaction (PCR) was carried out. PCR product size for the primer is 152bp. The following primer was used to execute the PCR.

Forward primer: 5′-AGAAGACCCCCCTCGGAACC-3′

Reverse primer: 5′-ATCTGGAGGAAGCGGTAGTG-3′

According to the protocol of Saxena et al. (2013), we constructed the PCR primer.

2.6.2 Polymerase Chain ReactionCondition for the TNF-α Gene

Taq polymerase was used to carry out the Polymerase Chain Reaction. Then added all the necessities for amplification of desired DNA and carried out the primary step of denaturing the DNA at 94oC for 5 minutes. Then repeated 35 cycles of denaturation; at 94ºC for 45 seconds. After that, the annealing step was done at 56ºC for 30 seconds, followed by an elongation step at 72ºC for 45 seconds, and lastly, a step of final elongation at 72ºC for 7 minutes. In a DNA thermal cycler, analyzed PCR assays (ASTEC). PCR condition was fixed using the protocol of Kim et al.(2017).

Table2.2:**Composition of the Reaction Mixture for PCR (15 µL)**

Name of the component	**Volume (µL)**
PCR-H_2O	3.6
5 X Green Buffer	3.0
Colorless Buffer	0.8
DMSO	3.0
d NTPs	0.3
Reverse Primer	0.6
Forward Primer	0.6
Go Taq Polymerase	0.1
Genomic DNA	3.0
Total	**15.00 (µL)**

2.6.3 Evaluation of PCR Product of the TNF-α Gene

A 2% agarose gel was used to check the amplification of the PCR product of fiveµL. A 100 bp DNA ladder was used to compare with the optional size of the PCR product. The amplified DNA

was stained with ethidium bromide solution. After that, we used UV light for visualizing amplified DNA over a gel image. The image was captured and documented.

2.6.4 RFLP Analysis of TNF-α Candidate Marker

By using a site-specific restriction enzyme (*Msp1*), analysis of TNF-α gene polymorphism at position G238A was done. By following the standard digestion protocol, performed restriction enzyme digestion. Candidate TNF-α variants disrupted the restriction enzyme site and was detected by restriction fragment length polymorphism (RFLP) assay.

2.6.5*Msp1* Restriction DigestionProtocol for the TNF-α Gene

PCR products were digested for up to 1 to 4 hours by using the *Msp1* enzyme at 37°C. Then imaged the Restriction Fragment Length Polymorphism product removed from gel electrophoresis by coloring with ethidium bromide stain. Afterward, it was noticed that three fragments of *Msp1* digested end product depending on the genotype; **GA** (hetero mutant): 152/133/19 bp; **GG**(homo normal): 133/19 bp, and **AA**(homo mutant): 152bp. The digestion was done in a reaction volume of 20 μL. The enzyme digestion protocol was as followed:

Table2.3: Composition of Reaction Mixture for *Msp1* DigestionbyRestrictionEnzyme

Name of the Component	**Volume (μL)**
PCR reaction mixture	5.0
PCR H2O	12.5
10 X Buffer	2.0
Msp1 enzyme(RE)	0.5
Total	**20.00(μL)**

2.7 IL 6 Gene Analysis

In this case, 597 A>G (rs1800797) was used as a candidate marker. The Fok1 restriction enzyme analyzed polymorphism. Candidate gene analysis was carried out by the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism method. In order to approximate the size of the fragment, a 100 bp ladder was used.

2.7.1 PCR of the IL6 Gene

By using 15 μLreaction volume to carry out the polymerase chain reaction with a primer of 527bp. The following primer was used to conduct PCR:

Forward primer: 5′-CTAAGTGGGCTGAAGCAGGT-3′

Reverse primer: 5′-CCAAGCCTGGGATTATGAAG-3′

According to the protocol of Saxena et al. (2013), we constructed the PCR primer.

2.7.2 PCR Condition of the IL6 Gene

Taq polymerase was used to carry out the Polymerase Chain Reaction. Then added all the necessities for amplification of desired DNA, and then carried out the primary step of denaturing the DNA at 94^{o}C for 5 minutes, and then repeated 35 cycles of denaturation; at 94^{o}C for 30 seconds. After that, the annealing step was done at 54^{o}C for 30 seconds, followed by an elongation step at 72^{o}C for 45 seconds, and lastly, a step of final elongation at 72^{o}C for 7 minutes. In a DNA thermal cycler, analyzed PCR assays (ASTEC). PCR condition was fixed using the protocol of Kim et al.(2017).

Table 2.4: Composition of the Reaction Mixture for PCR (15 µL).

Name of the component	Volume (µL)
PCR-H_2O	6.4
Colorless Buffer	3.0
DMSO	2.5
d NTPs	0.2
Reverse Primer	0.2
Forward Primer	0.2
Go Taq Polymerase	0.5
Genomic DNA	2.0
Total	**15.00 (µL)**

2.7.3 Evaluation of PCRProduct of the IL6 Gene

A 2% agarose gel was used to check the amplification of the five µL PCR product. A 100 bp DNA ladder was used to compare with the optional size of the PCR product. The amplified DNA was stained with ethidium bromide solution. After that, UV light was used for visualizing amplified DNA over the gel image. The image was captured and documented.

2.7.4 RFLP Analysis of IL6 Gene Candidate Marker

By using a site-specific restriction enzyme (*Fok1*), IL-6 gene polymorphism at position IL6-A597Gwas analyzed. By following the standard digestion protocol, restriction enzyme digestion was performed. Candidate IL-6 variants disrupted the restriction enzyme site and were detected by the restriction fragment length polymorphism (RFLP) assay.

2.7.5*Fok1* Restriction Digestion Protocol for the IL6 Gene

PCR products were processed for up to1 to 4 hours using the *Fok1* enzyme at 37°C. The Restriction Fragment Length Polymorphism product, which was removed from gel electrophoresis, was imaged by coloring with ethidium bromide stain. Afterward, it was noticed that three fragments of *Fok1*digested end product depending on the genotype; **AG**(heterozygous mutant): 527/461/66 bp; **GG** (homozygous mutant): 461/66 bp and **AA** (Homozygous normal): 527bp. The digestion was carried out in a reaction volume of 20 μL.

Table 2.5: Composition of Reaction Mixture for *Fok1* DigestionbyRestrictionEnzyme

Name of the Component	Volume (μL)
PCR reaction mixture	5.0
PCR H_2O	12.5
10 X Buffer	2.0
*Fok1*enzyme(RE)	0.5
Total	**20.00 (μL)**

2.8 IL 10 Gene Analysis

In this case, 592 C>A (rs1800872) was used as a candidate marker. Polymorphism was analyzed by the *Rsa1* restriction enzyme. Candidate gene analysis was carried out by the PCR-RFLP method. In order to approximate the size of the fragment, a 100 bp ladder was used.

2.8.1 PCR of the IL10 Gene

A polymerase chain reaction (PCR) was performed with a reaction volume of 15 μL. PCR product size for the primer is 241bp. The following primer was used to conduct PCR

Forward primer: 5′-TGGAAACATGTGCCTGAGAA-3′

Reverse primer: 5′-CCCCAACCTGGGATGAATAC-3′

PCR primers were constructed according to the protocol of Roorwakiwal et al. (2013).

2.8.2 PCR Condition of the IL10 gene:

Taq polymerase was used to carry out the Polymerase Chain Reaction. Added all the necessities for amplification of desired DNA; we carried out the primary step of denaturing the DNAat94^{o}C for 5 minutes, and then we repeated 35 cycles of denaturation; at 94^{o}C for 30 seconds. After that, the annealing step was done at 54^{o}C for 30 seconds, followed by an elongation step at 72^{o}C for 45 seconds, and lastly, a step of final elongation at 72^{o}C for 7 minutes. In a DNA thermal cycler, we analyzed PCR assays (ASTEC). PCR condition was fixed using the protocol of Hussain et al. (2013).

Table 2.6: Composition of the Reaction Mixture for PCR (15 µL)

Name of the component	Volume (µL)
PCR-H_2O	**6.4**
Colorless Buffer	**3.0**
DMSO	**2.5**
d NTPs	**0.2**
Reverse Primer	**0.2**
Forward Primer	**0.2**
Go Taq Polymerase	**0.5**
Genomic DNA	**2.0**
Total	**15.00 (µL)**

2.8.3 Evaluation of PCRProduct of the IL10 Gene

A 2% agarose gel was used to check the amplification of the fiveµL PCR product. A 100 bp DNA ladder was used to compare with the optional size of the PCR product. The amplified DNA was stained with ethidium bromide solution and, after that, used UV light for visualizing amplified DNA over a gel image. The image was captured and documented.

2.8.4 RFLP Analysis of IL10 Candidate Marker

By using a site-specific restriction enzyme (*Rsa1*), we analyzed IL-10 gene polymorphism at position IL10-C592A. By following the standard digestion protocol, performed restriction enzyme digestion. Candidate IL-10 variants disrupted the restriction enzyme site and were detected by restriction fragment length polymorphism (RFLP) assay.

2.8.5 *Rsa1* Restriction Digestion Protocol for the IL10 Gene

We processed PCR products for up to1 to 4 hours using*Rsa1* at 37°C. After that, it was imaged the restriction fragment length polymorphism product, which was removed from gel electrophoresis by coloring with ethidium bromide stain. Afterward, we noticed three fragments of *Rsa1* digested end product depending on the genotype; **CA**(heterozygous mutant): 241/166/75bp; **AA** (homozygous mutant): 241bp and **CC** (Homozygous normal): 166/75bp. The digestion was carried out in a reaction volume of 20 μL.

Table 2.7: Composition of Reaction Mixture for *Rsa1* Digestion by RestrictionEnzyme

Name of the Component	Volume (μL)
PCR reaction mixture	**5.0**
PCR H_2O	**12.5**
10 X Buffer	**2.0**
*Rsa1*enzyme(RE)	**0.5**
Total	**20.00(μL)**

2.9 Estimation of Lipid profile

2.9.1 Estimation of Total Cholesterol

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

2.9.1.1 Principle

After enzymatic hydrolysis and oxidation, the cholesterol was determined. In the presence of phenol and peroxidase, the predictor quinoneimine is formed from hydrogen peroxide and 4-aminoantiphyrine.

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

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

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

2.9.1.2 Reagents:

Table 2.8: Total Cholesterol Estimation Reagents

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)

2.9.1.3 Procedure

Serum and reagents were collected in a separate unit. They were arranged in a linear order. After that, each test's ID number was inserted into the AUTOLAB. In the Auto lab, 5 mL sample and 500 mL reagent were combined and incubated at 37^0C for 5 minutes. In a reaction cell or cup, the reaction took place. Within 60 minutes, the absorbance of the sample and the baseline against the reagent blank were estimated at 500 nm.

2.9.1.4 Calculation

The concentration of cholesterol in the sample was determined using the following formula and a software program:

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

2.9.2Estimation of Triglycerides

The enzymatic colorimetric (GPO-PAP) method was used to measure serum triglyceride in an auto-analyzer (Analyzer Medical System, Rome, Italy) with commercial Randox laboratories, UK kit (Trinder, 1969).

2.9.2.1 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 the catalytic influence of peroxidase.

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

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

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

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

2.9.2.2 Reagents

Table 2.9: 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)

2.9.2.3 Procedure

In a specific cup or cell, serum and reagents were collected. They were arranged in a linear order. After that, each test's ID number was inserted into the AUTOLAB. Within the AUTOLAB, 5 ml sample and 500 ml reagent were mixed and incubated for 5 minutes at 37 ^{0}C.

In the reaction cell, the reaction took place. Within 60 minutes, the absorbance of the sample and the baseline against the reagent blank were estimated at 500 nm.

2.9.2.4 Calculation

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

$$\text{Triglyceride concentration (mg/dL)} = \frac{A_{Sample}}{A_{S\,\tan dard}} \times \text{Concentration of standard.}$$

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

2.9.3.1 Principle

By adding a precipitating reagent (phosphotungstic acid-magnesium chloride) to serum, High-Density Lipoproteins (HDL) were isolated from chylomicrons, Very Low-Density Lipoproteins (VLDL), and Low-Density Lipoproteins (LDL). The cholesterol concentration in the HDL (high-density lipoprotein) fraction, which remained in the supernatant after centrifugation, was measured using an enzymatic colorimetric process using CHOD- PAP (Lopes-Verila et al., 1977)

2.9.3.2 Reagents

Both reagents for determining the levels of high-density lipoprotein-cholesterol in serum samples were obtained from RANDOX, UK.

Table 2.10: 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 (1.29mmol/L)

2.9.3.3 Procedure

In a centrifuge tube, samples (100µL) and precipitating reagents (250µL) were taken and homogenized by shaking in a vortex mixer. Then it was centrifuged at 4000rpm for 10minutes. 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, 45mg/dL) were prepared by diluting the stock standard solution of HDL-Cholesterol (50mg/dL). Standard HDL Cholesterol solutions (20µL) of each concentration were taken in the initial six micro-well of the plate. The first two wells were kept blank. Then the serum (20µL) was taken in the remaining micro-wells of the plate, and the working reagent (200µL) was added to all the wells. The mixture was then incubated for 5 minutes at 37ºC, and the absorbance of the solution was measured at 490nm with a micro-plate ELISA Reader (Bio-TekELx 808, USA).

Each material was subjected to two separate experiments. As a result, a calibration curve for the absorbance vs. concentrations of the reference solutions was obtained in comparison to a reagent null. The undisclosed cholesterol concentrations in the serum sample were calculated using the calibration curve, thus keeping the same mixing and incubation conditions as the regular solutions. Every day of the trial, the normal curve was drawn.

2.9.3.4 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-TekELx 808, USA).

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

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

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

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

2.10 Estimation of Serum Glucose

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

2.10.1Principle

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 form a red-violet quinoneimine dye as an indicator.

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

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

2.10.2 Reagents

Table 2.11: 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)

2.10.3Procedure

Without deproteinization, glucose was measured. Prior to estimation, the instrument was calibrated. In a separate cup, serum and reagent were collected. They were put in the AutoLab analyzer in sequential order (Analyzer Medical system, Rome, Italy). The Auto lab was set up to calculate glucose levels and operated using the following procedure:

5 mL sample and 500 mL reagent were combined and incubated for 10 minutes at 37° C. In the reaction cell, the reaction took place. Within 60 minutes, the absorbance of the sample and the baseline against the reagent blank were estimated at 500 nm.

2.10.4Calculation

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:

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

2.11Estimation of Glycosylated Hemoglobin (HbA_{1c})

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

2.11.1 Principle

The Bio-Rad VARIANT Hemoglobin A_{1c} program utilizes principles of ion-exchange high-performance liquid chromatography (HPLC) 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. The non-enzymatic glycation of HbA produces HbA_1c, the glycohemoglobin of interest, in two steps. The formation of an unstable aldimine (labile A1c, or pre-A1c) is the first step, which is a reversible reaction between the carbonyl group of glucose and the N terminal valine of hemoglobin's -chain. The development of labile A1c is proportional to the blood glucose level. Any of the labile A1c is transformed to a stable ketoamine, HbA_1c, during red blood cell circulation (Amadori rearrangement). The D-10 Hemoglobin A1c Program uses a cation exchange cartridge to chromatographically separate HbA1c. Separation is optimized to minimize interferences from hemoglobin variants, labile A1c, and carbamylated hemoglobin.

Table 2.12: 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 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.

2.11.2Procedure

Allow sample tubes to reach room temperature (15–30 °C) before conducting the assay, then gently invert the tube to thoroughly mix the sample. A simple preparation of the patient sample is required prior to the examination to hemolyze the blood and extract labile A1c. After diluting the samples with hemolysis reagent, they are incubated for a minimum of 30 minutes at 18-28^{o}C. The VARIANT's two dual-piston pumps deliver to the analytical cartridge a programmed buffer gradient of increasing ionic pressure. The hemoglobins are isolated based on their ionic interactions with the substance in the analytical cartridge, which is immediately infused with the prepared samples. The extracted hemoglobin is then passed through the filter photometer's flow cell, where differences in absorbance (415 nm) are measured; a background correction filter at 690 nm is used to correct for background variations. The raw data obtained from each study is reduced by a built-in integrator. For each cycle, a calibrator is examined for the purpose of fine-tuning the measurement parameters for HbA1c determination. The variations in absorbance are measured against the retention period in a chromatogram. Each chromatogram printout comes with a chart that lists each peak found, as well as the peak's relative percent and retention periods.

Pipet 1.5 mL Wash/Diluent Solution into a labeled 1.5 mL vial, followed by 5 mL whole blood sample to predilute. Fill the sample vial halfway with water and thoroughly mix it.

2.11.3 Calculation

The samples were run in the auto-analyzer. After analysis of the calibrator, the calibration response factor for HbA_{1c} is automatically calculated. Each sample receives a sample report as well as a chromatogram. The peak of A1c is shaded. The labile A1c and carbamylated peak areas are removed from the A1c peak area, which is determined using an exponentially modified Gaussian (EMG) algorithm.

2.12Estimation 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).

2.12.1Principle

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. In the coated well, an aliquot of a patient sample containing endogenous insulin is incubated with enzyme conjugate, which is a biotin-conjugated anti-insulin antibody. The unbound conjugate is washed away after incubation. Streptavidin Peroxidase Enzyme Complex binds to the biotin-anti-insulin antibody during the second incubation stage. The concentration of insulin in the sample is proportional to the amount of bound HRP (Horseradish peroxidase enzyme) complex. The strength of color produced after applying the substrate solution is proportional to the concentration of insulin in the subjects' sample.

2.12.2 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 preservatives.

6. **Substrate Solution:** Tetra-methyl-benzidine (TMB).
7. **Stop Solution:** contains 0.5 M H2SO4,
8. **Wash Solution:** 40X concentrated.

2.12.3 Wash Solution

To the 40X concentrated Wash Solution, add deionized water. To make a final volume of 1200 mL, dilute 30 mL of condensed Wash Solution with 1170 mL deionized water.

2.12.4 Procedure

In the frame holder, position the desired number of Microtiter wells. Fill sufficient wells with 25 l of each Norm, power, and sample with new disposable tips. Fill each well with 25 mL Enzyme Conjugate. In this stage, thoroughly mix for 10 seconds to ensure full mixing. Then, at room temperature, incubated for 30 minutes. After incubation, the contents of the wells were shaken out of the plate easily. Using diluted Wash Solution (400 L per well), rinse the wells three times. To affect the accuracy and precision of this assay, hit the wells sharply on absorbent paper to extract residual droplets. After that, pour 50 liters of Enzyme Complex into each well. Then, at room temperature, incubated for 30 minutes. Shake the contents of the wells vigorously once more. Using diluted Wash Solution (400 L per well), rinse the wells three times. To clear any remaining droplets, strike the wells hard on absorbent paper. Add 50 µL of Substrate Solution to each well. Again, incubate for 15 minutes at room temperature. Finally, stop the enzymatic reaction by adding 50 µL of Stop Solution to each well. 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.

2.12.5 Calculation

1. For each set of criteria, controls, and patient samples, calculate the average absorbance values.

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. This standard curve can be used to determine the concentration of the samples. Samples with concentrations greater than the highest normal must be diluted more or recorded as > 100 IU/mL. This dilution aspect must be taken into consideration when calculating concentrations.

2.13 Calculation of HOMA-IR, HOMA B%, Secretory HOMA, and VAI

HOMA-IR, HOMA B%, Secretory HOMA and VAI were calculated by the following formula respectively

2.13.1 HOMA-IR=fasting insulin (mU/lx fasting glucose (mmol/l/22.5

2.13.2 HOMA-B %=20XInsulin(mU/l)/{FBG(m. mol/l)-3.5

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

2.13.4 VAI

Formula for calculating visceral adiposity index (VAI)

For Male=WC (waist circumference)/39.68+(1.88XBMI}X(TG/1.03) X(1.31/HDL)

For Female=WC (waist circumference)/36.58+(1.89XBMI}X(TG/.81) X(1.52/HDL)

2.14 Anthropometric Measurements

Height was measured by a meter scale, and weight was measured using a calibrated weight machine by following the standard procedure.

Body Mass Indexes (BMI) of the subjects were calculated using the following formula.

$$\mathbf{BMI} = \frac{\boldsymbol{Weight\ (kg)}}{\boldsymbol{Height\ (m)^2}}$$

2.14.1 Waist Measurement

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

2.14.2 Hip Circumference

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

2.14.3 Waist Hip Ratio (WHR)

The Waist Hip Ratio was calculated using the standard formula.

2.15 Measurement of Blood Pressure

Blood pressure was measured in a sitting position, with the calf at the level of the heart. After 10 minutes of rest, a second reading was taken. Recorded Korotkoff 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-HIS).

2.16 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 as well as one-way ANOVA was carried out using Statistical Package for Social Science (SPSS) version 22. At the same time, a t-test was performed to analyze the relationship between lipid profile and type 2 diabetes. 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. The p-value of <0.05 was considered statistically significant.

3. RESULTS

The results of the study on the association of TNF-α, IL-6, and IL-10 gene polymorphisms with T2DM are presented in this chapter. The lipid profile of the study subjects was also presented. The study was conducted with two groups of subjects. These are the type 2 diabetes patient group (n=350) and control group (n=350) with the same age range. The number of male and female participants were the same (n=175) in both groups.

3.1 Anthropometric Characteristics of the Study Subjects

Table 3.1 displayed the anthropometric characteristics of the study subjects. There were significant ($p<0.001$) differences in mean systolic and diastolic blood pressure (SBP, DBP) among the groups. The value of systolic blood pressure was 126.44±15.84 mmHg in the diabetic group, and 117.01±15.46 mmHg in the control group, and the diastolic blood pressure (DBP) was 82.21±8.12 mmHg in the diabetic group and 78.63±8.89 mmHg in the control group, respectively.

Table 3.1: Anthropometric Characteristics of the Study Subjects

Variables	**Study Subjects (n=700)**	
	Control (n=350)	**T2DM (n=350)**
Age (year)	46.02 ± 5.43	47.04 ± 4.90
BMI (kg/m^2)	25.15±4.06	25.63±3.07
WHR	0.89±0.05	0.89±0.04
SBP (mmHg)	117.01±15.46	126.44±15.84**
DBP (mmHg)	78.63±8.89	82.21±8.12**

Values were presented as Mean ±SD; SBP= systolic blood pressure; DBP= Diastolic blood pressure; BMI= Body mass index; WHR= Waist hip ratio; p-value was obtained from individual sample t-test, **$p<0.001$; level of significance was set to $p<0.05$.

3.2 Socio-demographic Features of the Study Subjects

Table 3.2 showed the socio-demographic characteristics of the study subjects. Here, significant differences were found in education ($p<0.05$) and area of residence ($p<0.001$) among the two groups. This table showed that secondary and higher secondary levels of education were significantly ($p<0.05$) higher in the T2DM group compared to the control. This table also showed that T2DM subjects were significantly ($p<0.001$) higher in an urban area compared to the control group.

Table 3.2 Socio-demographic Features of the Study Subjects

Parameters	**Study Subjects (n=700)**	
	Control (n= 350) n (%)	**T2DM (n= 350) n (%)**
Education		
Primary	103 (29.42)	96 (27.42)
Secondary	108 (30.85)	149 (42.57) *
Higher Secondary	56 (16)	68 (19.40)*
Graduate/ Post graduate	83 (23.71)	37 (10.57)
Area of residence		
Urban	115 (32.85)	227 (64.85)**
Rural	235 (67.15)	123 (35.14)

p-value was obtained from the chi-square test; *p<0.05; **p<0.001, and the level of significance was set to p <0.05.

3.2.1: Family history of T2DM, Hypertension, and Behavioural Characteristics about the Study subjects

Table 3.3 showed the family history of T2DM, hypertension, and some habit-related information about the study population. The results suggested that the odds of developing diabetes from a family of diabetic history was 4.44 times more compared to a family without diabetic history. It also suggested that the odds of developing hypertension from a hypertensive family history was 6.81 times more compared to a family without hypertensive history. There were no significant

differences in exercise, smoking, and fast food-taking habits between the diabetic and control groups.

Table 3.3: Family History of T2DM, Hypertension, and Behavioural Characteristics about the Study Subjects

Parameters	Study Subjects (n=700)		OR (95% CI)	p-value
	Control (n=350) n (%)	T2DM (n= 350) n (%)		
Exercise/day				
No	38 (10.90)	36 (10.30)	1.06 (0.66-1.72)	>0.05
Yes	312 (89.10)	314 (89.70)		
Family History of DM				
No	224 (64)	100 (28.60)	4.44 (3.23-6.11)	<0.001
Yes	126 (36)	250 (71.40)		
Smoking				
No	307 (87.70)	321 (91.70)	0.65 (0.39-1.06)	>0.05
Yes	43 (12.30)	29 (8.30)		
Fast food taking habit/week				
No	105 (30)	111 (31.70)	0.92 (0.67-1.27)	>0.05
Yes	245 (70)	239 (68.30)		
Family History of Hypertension				
No	320 (91.42)	214 (61.14)	6.81(4.42-10.49)	<0.001
Yes	30 (8.60)	136 (38.85)		

Results were expressed as number (percentage); OR= Odds ratio; CI= Confidence interval; *p<0.001; Level of significance was set to p<0.05.

3.3: Biochemical Characteristics of the Study Subjects

3.3.1:Biochemical (Glycemic and Insulinemic) Characteristics of the Study Subjects

Table 3.4 showed biochemical data of the glycemic, insulinemic status of the study population. The Fasting Blood Glucose (FBG) levels were 8.77±3.0 (mmol/L) and 4.82±1.21 (mmol/L), 2 hours after

breakfast (ABF), 12.13±4.05 (mmol/L), 6.93±1.21(mmol/L), and HbA_1c% were 7.26±1.76 and 5.23±0.74 in the T2DM and control group, respectively. FBG, ABF, and HbA_1c% levels of the diabetic group were significantly ($p < 0.001$) higher than the control group. To investigate insulinemic status, fasting serum insulin was estimated by standardized method, and beta-cell function (HOMAB%), insulin resistance (HOMA IR), and insulin secretory capacity (Secretory HOMA) was calculated. The Fasting serum insulin level of the case group (23.22±15.73) (μU/L) was significantly ($p<0.001$) higher than control (16.03±10.79) (μU/L).On the other hand, the HOMA B% (101.34±26.12vs 309.12±47.23; $p<0.001$); HOMA-IR(9.19±7.62 vs 3.39±2.65; $p<0.001$), and secretory HOMA (103.01±102.79vs 357.04±101.42; $p<0.001$) were significantly lower in the diabetic group compared to control; whereas insulin was significantly higher (23.22±15.73 vs 16.03±10.79; $p<0.001$) in the T2DM group than in control.

Table 3.4: Biochemical (Glycemic and Insulinemic) Characteristics of the Study Subjects

Variables	Study subjects (n=700)	
	Control (n=350)	T2DM (n=350)
FBG (mmol/L)	4.82±1.21	8.77±3.00**
ABF (mmol/L)	6.93±1.21	12.13±4.05**
Fasting Insulin (μU/L)	16.03±10.79	23.22±15.73**
HbA_1c (%)	5.23±0.74	7.26±1.76**
HOMA IR	3.39±2.65	9.19±7.62**
HOMA-B%	309.12±47.23	101.34±26.12**
Secr HOMA	357.04±101.42	103.01±102.79**

Values were presented as Mean ±SD; FBG*:* Fasting blood glucose; ABF: 2 hours after breakfast; HOMA B%= Beta Cell Function; HOMA-IR: Homeostasis Model of Assessment Insulin Resistance; Secretory HOMA: Secretory Homeostasis Model of Assessment; p-value was obtained from individual sample t-test, **p<0.001; level of significance was set to p<0.05.

3.3.2:Biochemical (Lipidemic) Characteristics of the Study Subjects

Table 3.5: Biochemical (Lipidemic) Characteristics of the Study Subjects

Table 3.5 demonstrated the lipidemic status of the enrolled study subjects. The TG level was significantly ($p<0.001$) higher (189.45±106.31 mg/dL) in the diabetic group than that of the control group (142.57±87.28 mg/dL). The total cholesterol level was 184.92±42.86 mg/dL and 180.00±40.49 mg/dL in the diabetic and control groups, respectively. Although the cholesterol

level was lower in control subjects than that of T2DM, the difference was not statistically significant. The HDL level was significantly ($p<0.001$) lower in the T2DM group (38.20±7.34 mg/dL) compared to control (45.69±17.14 mg/dL); and LDL level was non significantly higher ($p>0.05$) in the T2DM group (113.42±42.03 mg/dL) compared to control group (105.38±79.31 mg/dL). VAI was significantly ($p<0.001$) higher in the T2DM group (3.93±2.72) compared to the control (2.60±2.22). There were no significant differences in LDL-C and total cholesterol levels between the two groups.

Table 3.5: Biochemical (Lipidemic) Characteristics of the Study Subjects

Variables	Study Subjects (n=700)	
	Control (n=350)	T2DM (n=350)
Triglycerides (mg/dL)	142.57±87.28	189.45±106.31**
Cholesterol (mg/dL)	180.00±40.49	184.92±42.86
HDL- Cholesterol (mg/dL)	45.69±17.14	38.20±7.34**
LDL- Cholesterol (mg/dL)	105.38±79.31	113.42±42.03
VAI	2.60±2.22	3.93±2.72**

Values were presented as Mean ±SD; HDL= High-Density Lipoproteins and LDL= Low-Density Lipoproteins. TC= Total Cholesterol; VAI: visceral adiposity index; SBP: systolic blood pressure; DBP: Diastolic blood pressure; p-value was obtained from individual sample t-test, **$p<0.001$; level of significance was set to $p<0.05$.

TNF – α GENE ANALYSIS (rsno-361525)

3.4 Determination of TNF- α Gene Genotype

Figure 3.1 showed the 152 base pair PCR product of the TNF- α gene after visualization on 3%agarose gel.

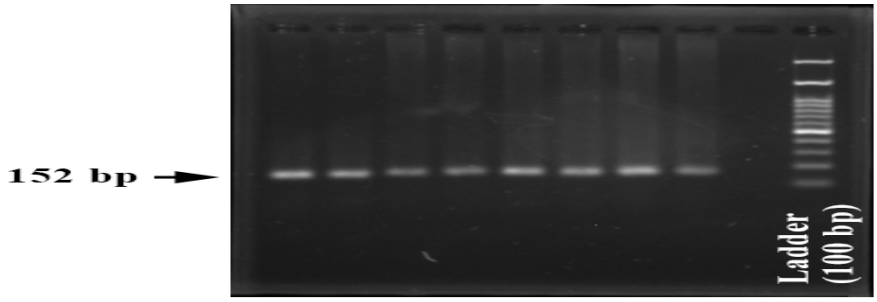

Figure 3.1: Agarose gel image of PCR product of TNF- α gene

3.4.1 RFLP Analysis of TNF- α gene Candidate Marker

In the present study, TNF 238G>A was used as a candidate marker. Polymorphism was analyzed by the MsP*1* restriction enzyme.

MsP*1*Digestion: PCR products (152bp) were digested for 1 to 4 hours with *Msp1* at 37°C. RFLP products were directly separated by electrophoresis in 3% agarose gel and visualized by ethidium bromide staining. *Msp1* digestion produces three fragments depending on the genotype; Heterozygous(Ht)variant genotype GA: 152bp,133bp, and 19 bp; Homozygous (Hz)wild genotype GG: 133bp and 19 bp; Homozygous (Hz) variant genotype AA: 152bp. Figure 3.2 presented the digestion pattern of the TNF- α gene. Restriction digestion produces GG variant: 133bp found in lane 3, AA variant:152bp found in lane 4; GA variant: 152bp and 133bp found in lane 5.

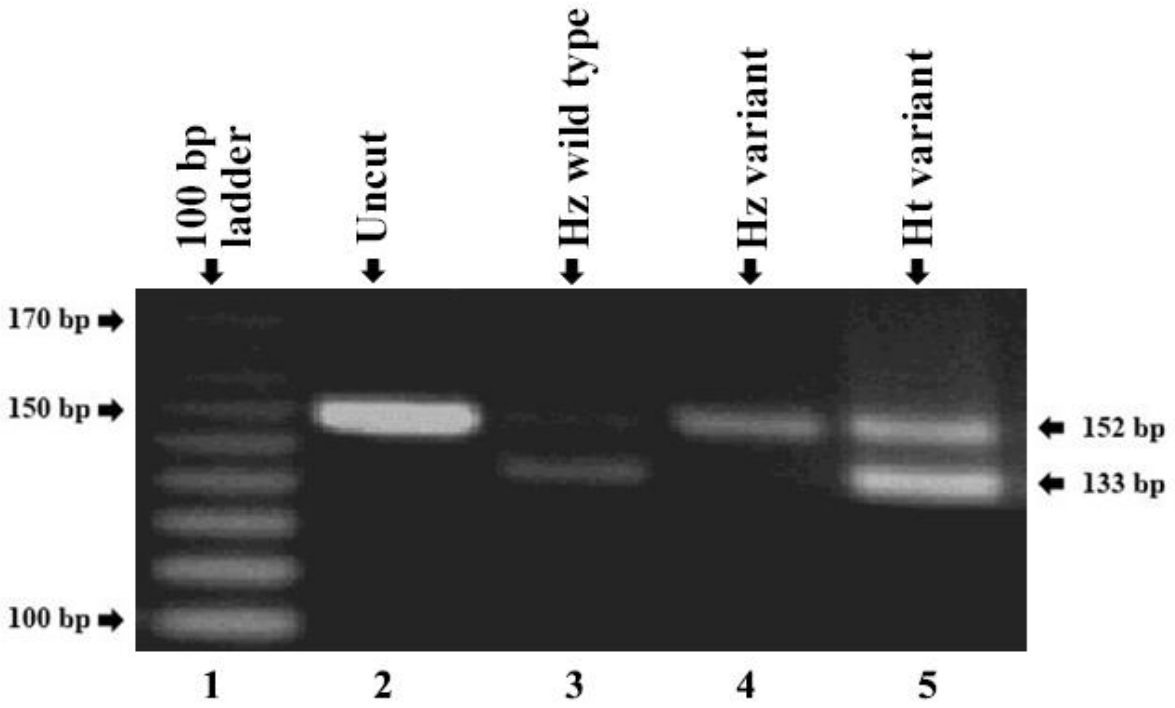

Figure 3.2: Agarose gel image of MsP1 digested PCR product of TNF- α gene

Table 3.6 showed that TNF-α G238A genotypic frequency follows the Hardy-Weinberg Equilibrium in the control group of the study subjects.

Table 3.6: Hardy- Weinberg Equilibrium Compares Genotype Frequencies in T2DM and Control for the TNF α gene.

Hardy Online Calculator	Study Subjects (n=700)					
	T2DM (n= 350) TNF αGenotype			Control (n=350) TNF αGenotype		
	GG	GA	AA	GG	GA	AA
Observed value	201	95	54	225	105	20
Expected value	176.435	144.13	29.435	220	114	15
Chi square value		40.66			2.62	

Table 3.7 showed the frequency distribution of TNF-α (G238A) genotypes and the risk of diabetes in the study subjects. The percentages of GG, GA, and AA genotypes at 238 positions of TNF alpha gene were 64%, 30%,6%, respectively, in the control group, while in the T2DM group, it was 57%, 27%, and 16%, respectively. Homozygous mutant variant (AA) of TNF alpha genotype had shown significant ($p<0.001$) association with T2DM, and odds of the homozygous mutant variant (AA) was 3.02 times more likely to develop the T2DM compared to homozygous normal (GG). Minor allele frequency was significantly ($p<0.001$) higher in T2DM than in control.

Table 3.7: Genotype Distributions and Allele Frequency of TNF-α (G238A) Polymorphism

Genotype	Study Subjects (n=700)		OR (95% CI)	p-value
	Control (n=350) n (%)	T2DM(n=350) n (%)		
GG	225 (64)	201 (57)	1(Ref)	
GA	105 (30)	95 (27)	1.01 (0.72 -1.41)	>0.05
AA	20 (6)	54 (16)	3.02 (1.75 - 5.22)	<0.001
Allele	**n, frequency**			
G	555 (79)	495 (71)	1.58 (1.24 - 2.02)	<0.001
A	145 (21)	205 (29)		

Results were expressed as number (percentage); The level of significance was set to $p<0.05$; OR=Odds ratio; CI=Confidence interval; GG=homozygous normal; GA=Heterozygousmutant, and AA= homozygous mutant.

Figure 3.3 showed that homozygous wild genotype GG carrying individuals in T2DM and control group were 201 and 225, respectively. Heterozygous mutant GA genotype carrying individuals in T2DM were 95 and 105 in the control group, whereas homozygous mutant genotype AA carrying individuals was 54 in T2DM and 20 in the control group.

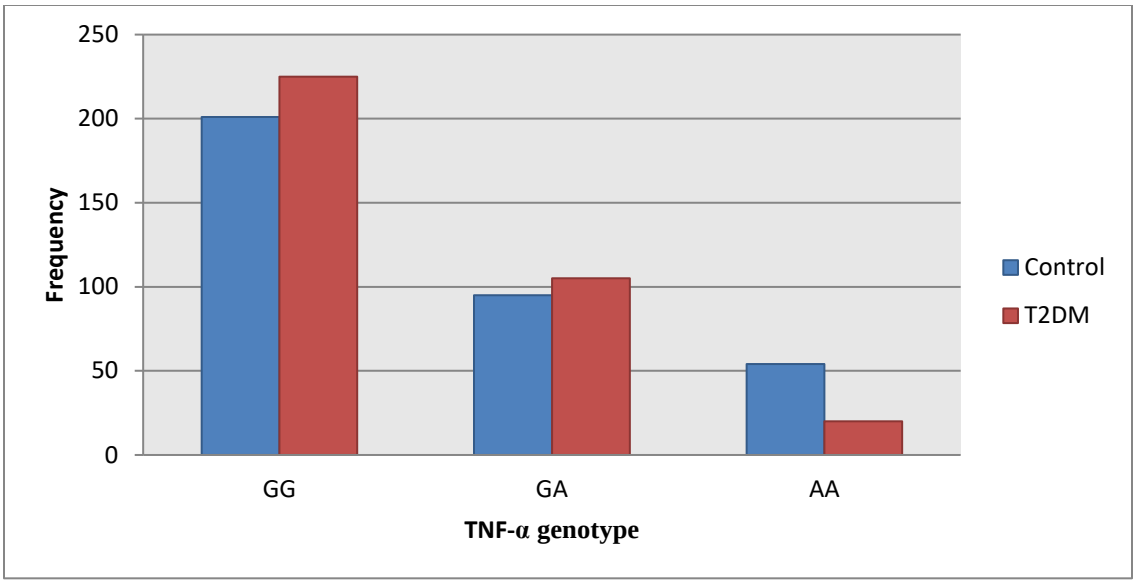

Figure 3.3: Distribution of TNF-α (G238A) Genotype in the study subjects (n=700)

Table 3.8 showed the binary logistic regression analysis of TNF-α G238A genotypes in dominant (GG versus GA+AA) and the recessive model (AA versus GG+GA). Here, GG versus the combination of GA and AA genotypes were considered the dominant model. AA versus the combination of GG genotype and GA genotypes were considered the recessive model collectively. It was found that the frequency of (GG+GA) genotype in the recessive model was significantly ($p<0.05$)correlated with T2DM, and the odds ratio was 66% less likely to develop T2DM compared to the control AA genotype where 95%CI was (0.19 -0.57).

Table 3.8: Binary Logistic Regression Analysis of TNF-α (G238A) Polymorphism in Dominant and Recessive model

Genotype	Study Subjects (n=700)		OR (95% CI)	p-value
	Control (n=350) n (%)	T2DM (n=350) n (%)		
Dominant model (GG vs GA+ AA)				
GG	225 (64)	201 (57)	1 (Ref)	
GA+AA	125 (36)	149 (43)	1.33 (0.98- 1.81)	>0.05
Recessive model (AA vs GG+GA)				
AA	20 (6)	54 (15)	1 (Ref)	
GG+GA	330 (94)	296 (85)	0.33 (0.19 -0.57)	<0.05

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; GG= genotype homozygous normal; GA= heterozygous mutant, and AA= homozygous mutant.

Table 3.9 showed the frequency distribution of TNF-α G238A genotypes in male participants and the risk of diabetes. Here only homozygous (AA) mutant variants showed significant (p<0.001) association and odds of the homozygous mutant variant (AA) was 4.12 times more likely to develop T2DM compared to normal male GG genotype where 95%CI= (1.94 - 8.74).

Table 3.9: Distribution of TNF-α (G238A) Genotypes in Male Study Subjects

Genotype	Study Subjects (n=300)		OR (95% CI)	p-value
	Control (n=175) n (%)	T2DM (n=175) n (%)		
GG	111 (63)	97 (55)	1 (Ref)	
GA	54 (31)	42 (24)	0.89 (0.55 - 1.45)	>0.05
AA	10 (6)	36 (20)	4.12 (1.94 - 8.74)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; GG= genotype homozygous normal; GA= heterozygous mutant, and AA= homozygous mutant.

Table 3.10 showed the frequency distribution of TNF-αG238A genotypes in female participants and the risk of type 2 diabetes. Here both heterozygous mutant variant (GA) and homozygous mutant AA showed no significant association with T2DM.

Table 3.10: Distribution of TNF-α (G238A) Genotypes in Female Study Subjects

Genotype	StudySubjects (350)		OR (95% CI)	p-value
	Control (n= 175) n (%)	T2DM (n= 175) n (%)		
GG	114 (65)	104 (59)	1 (Ref)	
GA	51(29)	53 (31)	1.13 (0.71-1.81)	>0.05
AA	10 (6)	18 (10)	1.97 (0.87-4.46)	>0.05

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; GG= genotype homozygous normal; GA= heterozygous mutant, and AA= homozygous mutant.

Table 3.11 showed the association of age differences in female participants with TNF-α G238A polymorphism. It was found that the heterozygous mutant (GA) variants of all age groups showed a significant (p<0.001) association with the risk of T2DM.

Table 3.11: Association of TNF-α (G238A) Polymorphism with Category of Female Age Group

	Study Subjects (n=350)				
(Year)	Genotype	Control (n=175)	T2DM (n=175)	OR (95% CI)	p-value
30-40	GG	38	16	1(Ref.)	
	GA	01	19	45.13 (5.56-366.50)	<0.001
	AA	01	03	7.12 (0.69 –73.81)	>0.05
41- 50	GG	95	52	1(Ref.)	
	GA	2	30	27.40 (6.29-119.30)	<0.001
	AA	2	9	8.22 (1.71 –39.49)	<0.05
51- 60	GG	32	25	1(Ref.)	
	GA	2	14	8.96 (1.86 – 43.14)	<0.05
	AA	2	7	4.48 (0.85 –23.48)	>0.05

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; GG= genotype homozygous normal; GA= heterozygous mutant, and AA= homozygous mutant.

According to the history of hypertension (Table 3.12), TNF-α G238A genotypic showed significant (p<0.001) association with T2DM for homo-mutant variant AA in negative history of

hypertension and odds having AA genotype was 3.59 times more likely to develop T2DM compared to GG genotype (OR=3.59, 95%CI=1.97– 6.55).

Table 3.12: Distribution of TNF-alpha (G238A) Genotype according to History of Hypertension

History of Hypertension	Genotype	Study Subjects (n=700)		OR(95% CI)	p-value
		Control (n=350)	T2DM (n=350)		
Yes	GG	16	73	1 (Ref.)	
	GA	12	47	0.85 (0.37–1.96)	>0.05
	AA	2	17	1.86 (0.39-8.88)	>0.05
No	GG	209	126	1 (Ref.)	
	GA	93	48	0.85 (0.56- 1.29)	>0.05
	AA	18	39	3.59 (1.97– 6.55)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; GG= genotype homozygous normal; GA= heterozygous mutant, and AA= homozygous mutant.

Table 3.13 showed the distribution of TNF-alpha (G238A) genotype according to the family history of diabetes. According to the family history of diabetes, homozygous mutant variant AA was significantly(p<0.05) correlated with T2DM, and odds of homozygous mutant AA variant was 2.95 times more likely to develop T2DM compared to the GG genotype in the positive family history of T2DM, and 95%CI was (1.37– 6.37).

Table 3.13: Distribution of TNF-alpha (G238A) Genotype according to the Family History of Diabetes

Family History of DM	Genotype	Study subjects (n=750)		OR(95% CI)	p-value
		Control (n=350)	T2DM (n=350)		
Yes	GG	81	134	1 (Ref.)	
	GA	35	68	1.17 (0.71-1.92)	>0.05
	AA	09	44	2.95 (1.37– 6.37)	<0.05
No	GG	144	66	1 (Ref.)	
	GA	69	27	0.853 (0.50- 1.45)	>0.05
	AA	12	11	2.00 (0.83–4.76)	>0.05

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; GG= genotype homozygous normal; GA= heterozygous mutant, and AA= homozygous mutant.

Table 3.14 showed the distribution of TNF-alpha (G238A) genotype according to the category of BMI and demonstrated a significant ($p<0.05$)association with T2DM for homozygous mutant variant AA genotype in normal and overweight BMI and odds having homozygous mutant AAvariant were 2.68 times and 4.34 times more likely to develop T2DM compared to control GG variant in the average body weight and overweight group (95% CI=1.28 – 5.60 and 1.59-11.80) respectively.

Table 3.14: Distribution of TNF-alpha (G238A) Genotype according to the Category of BMI

Category of BMI	Genotype	Study Subjects(n=700)		OR (95% CI)	p-value
		Control (n=350)	T2DM (n=350)		
Normal	GG	106	73	1 (Ref.)	
	GA	52	39	1.08 (0.65-1.81)	>0.05
	AA	13	24	2.68 (1.28– 5.60)	<0.01
Overweight	GG	92	106	1 (Ref.)	
	GA	44	44	0.86 (0.52-1.43)	>0.05
	AA	5	25	4.340 (1.59-11.80)	<0.05
Obese	GG	27	21	1 (Ref.)	
	GA	9	12	1.71 (0.60- 4.82)	>0.05
	AA	2	6	3.85 (0.70-21.10)	>0.05

Results were expressed as number (percentage); The level of significance was set to $p<0.05$; OR=Odds ratio; CI=Confidence interval; GG= genotype homozygous normal; GA= heterozygous mutant, and AA= homozygous mutant.

Table 3.15 showed the distribution of TNF alpha (G238A) genotype according to their smoking habit and showed a significant ($p<0.001$) association with heterozygous mutant GA genotype in the smoker group, whereas the significant ($p<0.001$) association was also found for homozygous mutant variant AA in non -smoker group.

Table 3.15: Distribution of TNF alpha (G238A) Genotype according to Their Smoking Habit

Smoking Status	Genotype	Study subjects (n=700)		OR (95% CI)	p-value
		Control(n=350)	T2DM (n=350)		
Smoker	GG	9	22	1 (Ref.)	
	GA	28	7	0.10 (0.03 - 0.31)	<0.001
	AA	6	5	0.34 (0.08 -1.40)	>0.05
Non-Smoker	GG	216	183	1 (Ref.)	
	GA	77	88	1.34 (0.93 -1.94)	>0.05
	AA	14	45	3.79 (2.01 - 7.13)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; GG= genotype homozygous normal; GA= heterozygous mutant, and AA= homozygous mutant

Table 3.16 showed the distribution of TNF alpha (G238A) genotype according to HBA_1C status and significant (p<0.01)association was found with T2DM for homozygous mutant variant AA genotype in HBA_1C <6.5% and odds having AA variant was 3.15 times more likely to develop T2DM compared to control GG variant (95% CI=1.63 – 6.10).

Table 3.16: Distribution of TNF alpha (G238A) Genotype according to HBA_1C Status

HBA_1C	Genotype	Study Subjects (n=700)		OR (95% CI)	p-value
		Control (n=350)	T2DM(n=350)		
≤6.5%	GG	221	77	1 (Ref.)	
	GA	100	38	1.09 (0.06 - 1.71)	>0.05
	AA	20	22	3.15 (1.63 – 6.10)	<0.01
≥6.6%	GG	4	123	1 (Ref.)	
	GA	3	57	0.61 (0.13 – 2.85)	>0.05
	AA	2	33	0.53 (0.09 - 3.05)	>0.05

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; GG= genotype homozygous normal; GA= heterozygous mutant, and AA= homozygous mutant

Table 3.17 showed the glycemic, insulinemic status according to TNFα G238A genotype in both T2DM and control groups. It showed no significant association with fasting blood glucose, 2 hours after breakfast, serum insulin, HBA_1C, HOMA-IR, HOMA B%, and Secret-HOMAin T2DM subjects, whereas, in control, there was a significant ($p<0.05$)association of genotypes found for HOMA B% and Secret-HOMA.

Table 3.17: Study of Glycemic and Insulinemic Biomarkers According to TNF-α (G238A) Genotype

Variables	Study Subjects (n=700)					
	T2DM (n=350) TNF-α Genotype			Control (n=350) TNF-α Genotype		
	GG (n= 200)	GA (n=95)	AA (n=55)	GG (n= 210)	GA (n=55)	AA (n=85)
FBS (mmol/L)	8.80 ±3.01	8.40±2.70	9.11 ±3.48	4.90±1.14	4.54±1.15	4.77±1.39
ABF (mmol/L)	12.11±3.90	11.71±3.70	12.95±4.88	7.02±1.18	6.86±1.23	6.72±1.26
HbA_1C (%)	7.27 ± 1.70	7.60 ± 1.71	7.22±1.90	5.20±0.70	5.21±0.84	5.36±0.75
Insulin(µU/L)	24.63 ±16.02	22.04±13.60	20.14±13.60	15.96±11.36	18.22±12.10	14.80±7.91
HOMA IR	9.80 ± 8.20	8.32 ± 6.02	8.32±7.54	3.47.03±9.87	3.59±2.75	3.03±1.92
HOMAB%	88.04±45.02	112.01±9.00	128.02±23.01	360.03±57.02	443.01±153.23	101.05±83.04*
Secrt-HOMA	85.01±54.03	124.03±12.02	127.04±17.03	416.67±66.04	500.23±177.14	117.56±96.23*

Results were presented as mean ± standard deviation (SD). Differences were considered significant at $p<0.05$;*=$p<0.05$: HOMA B%= expressed Beta Cell Function; HOMA S%= expressed Beta Cell Sensitivity; HOMA-IR: expressed Homeostasis Model of Assessment - Insulin Resistance; Secretory HOMA: Secretory Homeostasis Model of Assessment; HbA_1c= glycated hemoglobin; FBS= fasting blood sugar; ABF= 2 hours after breakfast.

Figure 3.4 showed a comparison of HOMA-IR level among GG, GA, and AA variant in T2DM subjects and showed positive skewness of data in homozygous normal GG genotype and homozygous mutant variant AA genotype. As shown in figure 3.4, the HOMA-IR level in the diabetes group was not significantly ($p>0.05$) different among GG, GA, and AA variant.

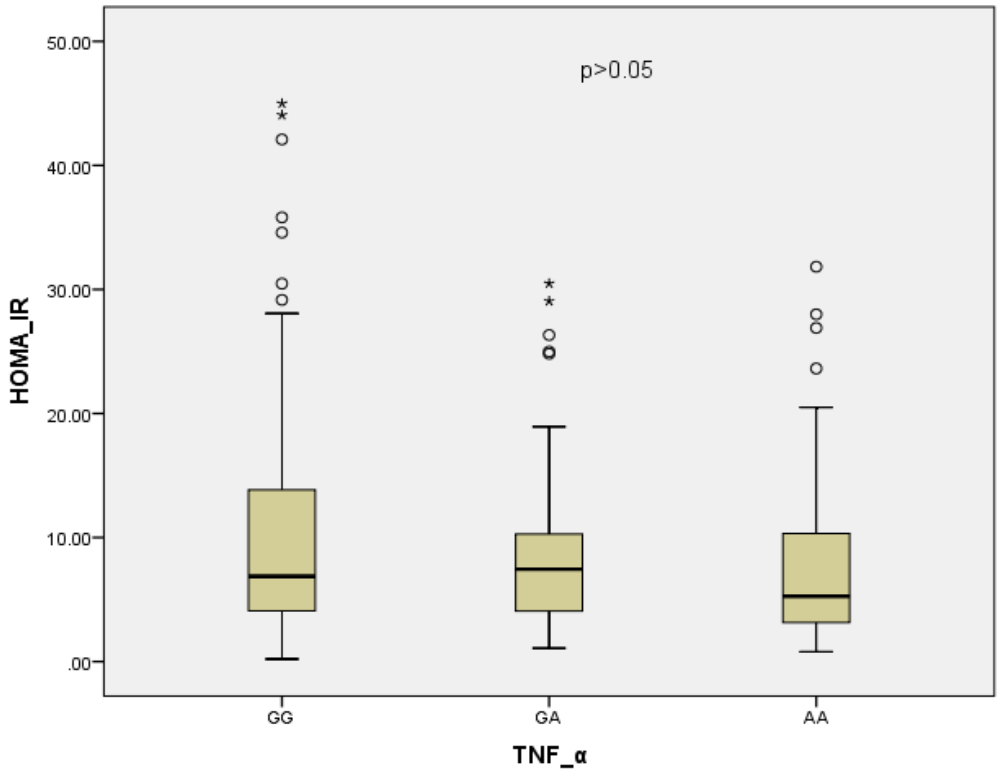

Figure 3.4: **Comparison of HOMA-IR level among GG, GA and AA variant in T2DM Subjects**

Figure 3.5 showed a comparison of serum insulin level among GG, GA, and AA variant in T2DM subjects and showed positive skewness of data in homozygous normal GG genotype and homozygous mutant variant AA genotype. As shown in figure 3.5, the serum insulin level in the diabetes group was not significantly ($p>0.05$) different among GG, GA, and AA variant.

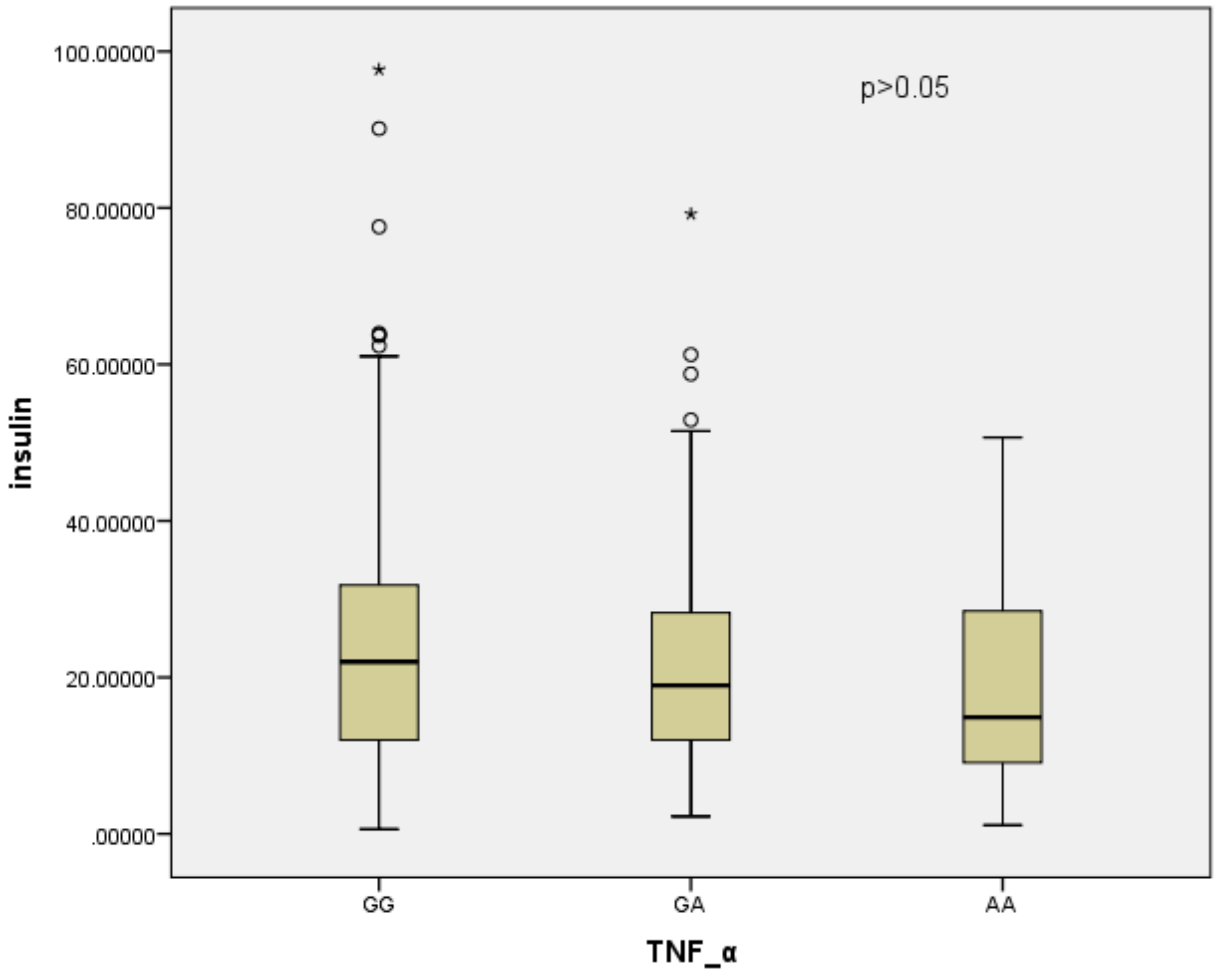

Figure 3.5: Comparison of serum insulin level among GG, GA, and AA variant in T2DM subjects

Table 3.18 showed the lipidemic status according to the TNF alpha G/A genotype in both T2DM and control subjects. It showed no significant distribution with serum cholesterol, triglyceride, high-density lipoprotein, low-density lipoprotein, and visceral adiposity index in T2DM and control group separately.

Table 3.18: Study of Lipidemic Status According to TNF-α (G238A) Genotype in the Study Subjects

Variables	Study Subjects (n=700)					
	T2DM (n=350)			Control (n=350)		
	TNF-α Genotype			TNF-α Genotype		
	GG (n=200)	GA (n=95)	AA (n=55)	GG (n=210)	GA (n=55)	AA (n=85)
Cholesterol (mg/L)	128.78±43.30	183.63±95.98	182.27±35.67	176.83±10.21	200.19±15.27	174.75 ±97.20
TG (mg/dL)	196.33±115.02	190.69±45.69	174.42±86.58	140.08±82.70	140.46±78.40	150.11±102.80
HDL-C(mg/dL)	38.08 ±7.04	39.11 ±7.27	37.09 ±8.34	45.99 ±17.27	42.30 ±17.52	47.14 ±16.48
LDL-C (mg/dL)	112.48±42.90	113.25±42.05	117.12±40.50	104.28±74.30	113.11±87.70	103.09 ±86.84
VAI	4.07±2.91	3.87±2.46	3.52±2.41	2.52±2.12	2.82±2.17	2.64±2.50

Results were presented as mean ± standard deviation (SD). Groups of data were compared using a one-way ANOVA test. The p-value was<0.05 taken as the significance level; HDL-C=High Density Lipoprotein; LDL-C=Low Density Lipoprotein; VAI= Visceral Adiposity Index; TG= Triglyceride; *=p<0.05.

Table 3.19 showed the association of TNF-α G238A polymorphism with glycemic parameters in the study population and showed all polymorphic variants (GG, GA, AA) of TNF- α G238A polymorphism had a significant ($p<0.05$) association with fasting blood glucose, 2hours after breakfast and were non significantly correlated withHBA_1C, fasting serum insulin, the homeostatic model of insulin resistance (HOMA-IR), HOMA B% and Secret -HOMA.

Table 3.19: Association of TNF-α(G238A) Polymorphism with Glycemic Parameters in the Study Subjects (n=700)

Variables	TNF-α Genotype	TNF-α Genotype	F- value	p-value
FBS[a,d] (mmol/L)	GG	GA AA*		
	GA	GG AA*	3.57	<0.05
ABF[a,d] (mmol/L)	GG	GA AA*		
	GA	GG AA*	4.59	<0.05
HBA_1C [a, d] (%)	GG	GA AA		
	GA	GG AA	1.37	>0.05
insulin[a,d] (µU/L)	GG	GA AA		|
	GA	GG AA	0.15	>0.05
HOMA-IR[a, d]	GG	GA AA		
	GA	GG AA	0.65	>0.05
HOMAB %[bc]	GG	GA AA		
	GA	GG AA	0.45	>0.05
secret_HOMA[b,c]	GG	GA AA		
	GA	GG AA	0.37	>0.05

Here, the a=Welch ANOVA test, since data violated homogeneity, b=One-way ANOVA Test, since data confirmed homogeneity, and c=LSD test since data confirmed homogeneity, d=Dunnett Test since data violated homogeneity. *= p<0.05; The p-value was<0.05 taken as the significance level.

Table 3.20 showed the association of TNF alpha G238A polymorphism with lipidemic and clinical parameters in the study population. The polymorphic variants (GG, GA, AA) of TNF alpha G238A polymorphism were significantly (p<0.05) correlated with waist-hip ratio, serum cholesterol, LDL-C, HDL-C, and diastolic blood pressure. Moreover, the polymorphic variants (GG, GA, AA) of TNF-α G238A polymorphism were not significantly correlated with age, body mass index, triacylglycerol, systolic blood pressure, visceral adiposity index.

Table 3.20: Association of TNF-α(G238A) Polymorphism with Lipidemic and Clinical Parameters in the Study Population

Variables	Genotype	Genotype	F -value	p-value
AGE[b,c] (years)	GG	GA AA		
	GA	GG AA*	2.64	>0.05
BMI[b,c] (Kg/m2)	GG	GA AA		
	GA	GG AA	0.22	>0.05
SBP[b,c] (mm of Hg)	GG	GA AA		
	GA	GG AA	2.67	>0.05
DBP[b,c] (mmHg)	GG	GA* AA		
	GA	GG* AA	3.69	<0.05
TG[b,c] (mg/dL)	GG	GA AA		
	GA	GG AA	1.81	>0.05
visceral adiposity index[b,c]	GG	GA AA		
	GA	GG AA	1.37	>0.05
HDL[a,d] (mg/dL)	GG	GA AA*		
	GA	GG AA*	4.79	<0.01
LDL[a,d] (mg/dL)	GG	GA AA		
	GA	GG* AA	4.36	<0.05
Cholesterol[a,d] (mg/dL)	GG	GA** AA		
	GA	GG** AA	8.63	<0.001
WHR[a,d]	GG	GA* AA		
	GA	GG* AA	10.50	<0.001

Here, the a=Welch ANOVA test, since data violated homogeneity, b=One-way ANOVA Test, since data confirmed homogeneity, and c=LSD test since data confirmed homogeneity, d=Dunnett Test since data violated homogeneity. The p-value <0.05 was significant; *=POST HOC p<0.05;**=POST HOC p<0.001; SBP=systolic blood pressure; DBP= Diastolic blood pressure; TG= Triglyceride; HDL=High density lipoprotein; LDL=Low density lipoprotein; WHR= Waist hip ratio.

INTERLEUKIN -SIX (IL-6) GENE ANALYSIS

(rs no1800797)

3.5 Determination of IL-6 Gene Genotype

Figure 3.6 showed the 527 base pair PCR product of the IL-6 gene after visualisation on 2% agarose gel.

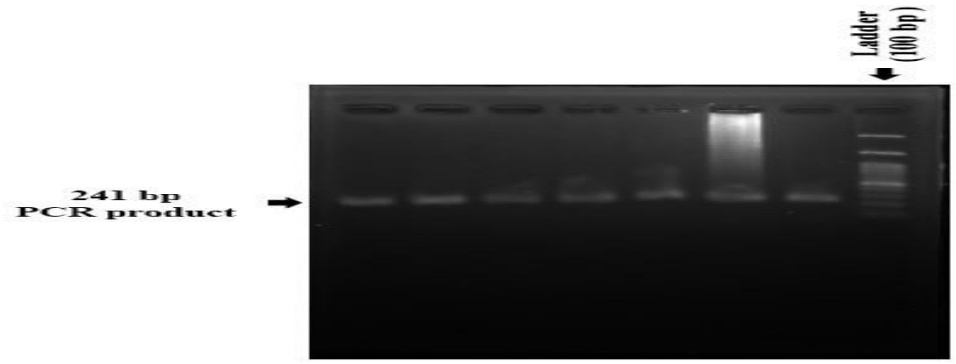

Figure 3.6:Agarose gel image of PCR product of IL6 gene

3.5.1 RFLP analysis of IL6 gene candidate marker

In the present study, IL-6 597A>G was used as a candidate marker. The Fok*1* restriction enzyme analyzed polymorphism.

***Fok1* Restriction digestion:** PCR products (527bp) were digested for 1 to 4 hours with *Fok1* at 37°C. RFLP products were directly separated by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining. *Fok1* digestion produces three fragments depending on the genotype; Heterozygous (Ht)variant genotype AG: 527bp,461bp, and 66 bp; Homozygous (Hz)variant genotype GG: 461bp and 66 bp; Homozygous (Hz) wild genotype AA: 527bp. Figure 3.7 presented the digestion pattern. Lane 2, 6,9 showed an AA variant: 527 bp; lane 4 showed an AG variant: 527bp and 461bp; lane 11 showed a GG variant:461bp and 66bp.

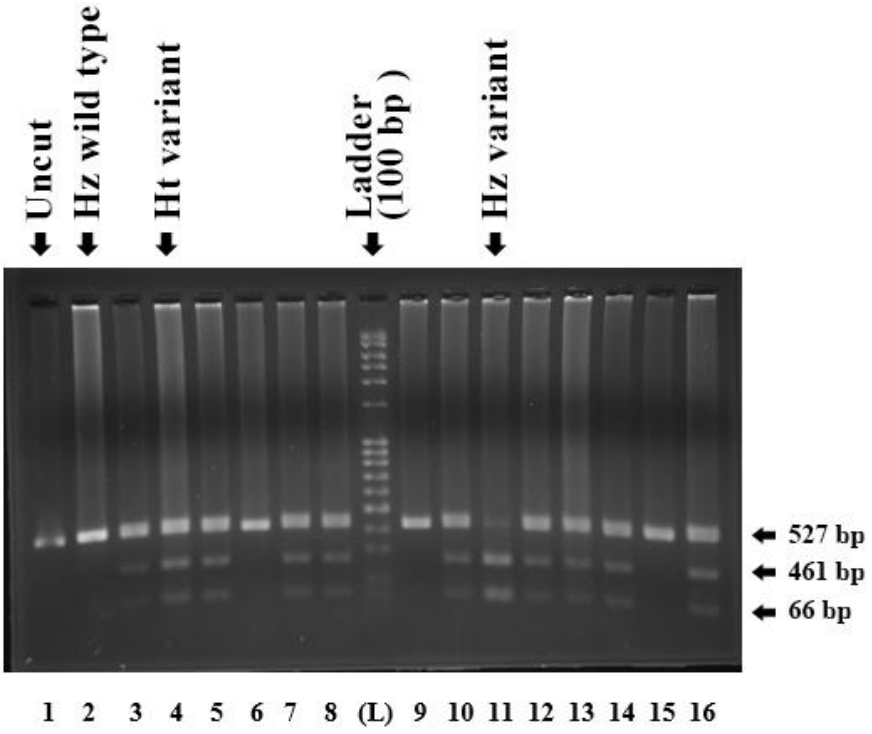

Figure 1.7: Agarose gel image of Fok1 digested PCR product of IL6 gene

In Table 3.21, it was found that IL-6 (A597G) genotypic frequency followed the Hardy-Weinberg Equilibrium in the control group.

Table 3.21: Hardy-Weinberg Equilibrium for Comparison of Genotype Frequencies in T2DM and control for IL-6 (A 597G) Gene Polymorphism

Hardy-Online Calculator	Study Subjects (n=700)					
	T2DM (n= 350) IL-6Genotype			Control (n=350) IL-6Genotype		
	AA	AG	GG	AA	AG	GG
Observed value	173	20	157	230	103	17
Expected value	95	175	80	226	110	13
Chi-square value		274			1.5	

Table 3.22 showed the genotype distributions and allele frequency of IL-6 (A597G) Polymorphism. Homozygous mutant variant (GG) of the IL-6 genotype showed a significant ($p<0.001$)association with T2DM where odds of homozygous mutant GG was 12.28 times more

likely to develop T2DM compared to control homozygous normal AA genotype. Minor allele frequency was significantly ($p<0.001$) higher in T2DM than in control.

Table 3.22: Genotype Distributions and Allele Frequency of IL-6 (A597G) Polymorphism

Genotype	Study Subjects (n=700)		OR (95% CI)	p-value
	Control (n=350) n (%)	T2DM(n=350) n (%)		
AA	230 (66)	173 (49)	1 (Ref)	
AG	103 (29)	20 (5)	0.26 (0.15- 0.43)	<0.001
GG	17 (5)	157 (46)	12.28 (7.16- 21.03)	<0.001
Allele	**(n, Frequency)**			
A	563 (80)	366 (52)	3.75 (2.95 – 4.76)	<0.001
G	137 (20)	334 (48)		

Results were expressed as number (percentage); The level of significance was set to $p<0.05$; OR=Odds ratio; CI=Confidence interval; AA= genotype homozygous normal; AG= heterozygous mutant, and GG= homozygous mutant.

Figure 3.8 showed the distribution of the IL-6 (A597G) genotype in the study population. This Bar diagram showed that homozygous wild genotype AA carrying individuals in T2DM and control group were 173 and 230, respectively. Heterozygous mutant variant AG genotype carrying individuals in T2DM were 20 and 103 in the control group, whereas homozygous mutant variant genotype GG carrying individuals was 157 in T2DM and 17 in the control group.

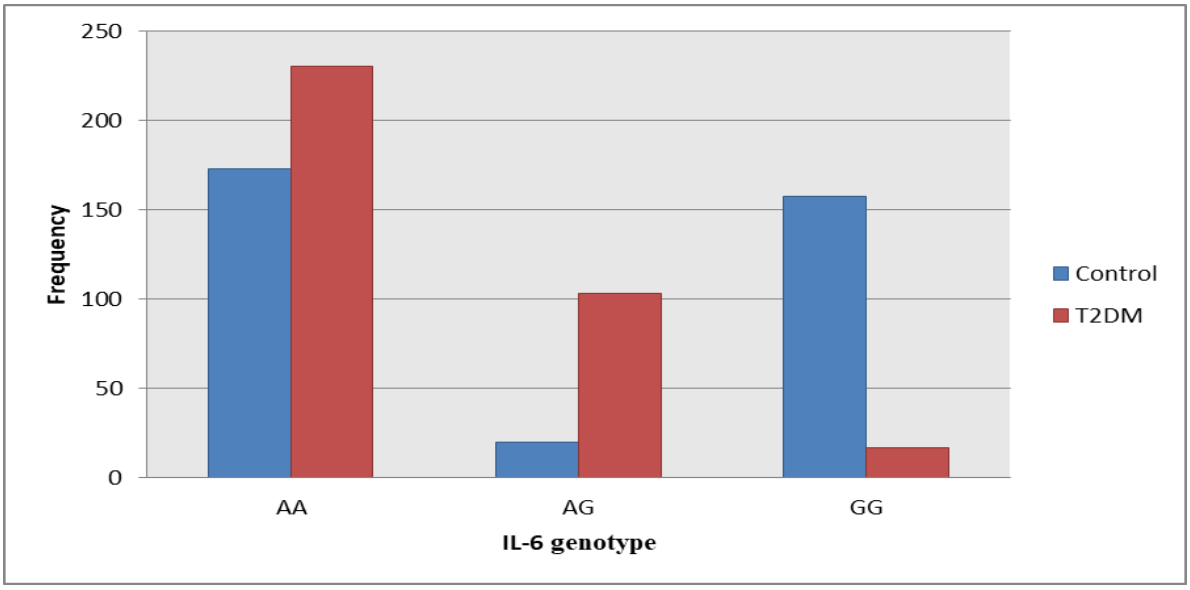

Figure 3.8: Distribution of IL6 (A597G) Genotype in the study population (n=700)

Table 3.23 showed the binary logistic regression analysis of IL-6 A597G genotypes in the dominant and recessive models. Here, AA versus the combination of AG and GG genotype was considered the dominant model. GG versus the combination of AA genotype and AG genotype were considered recessive models collectively. It was also found that in the dominant model, the frequency of AG+GG was significantly($p<0.001$) correlated with T2DM, and the odds ratio was 1.96 times more likely to develop T2DM in the cases of having AG or GG genotype compared to homozygous wild AA genotype where 95%CI was (1.45 – 2.66). It was found that in the recessive model, the frequency of AA+AG was also a significant($p<0.001$) association with T2DM; the odds ratio was 93% times less likely to develop T2DM in the cases of having AA or AG genotype compared to the GG genotype.

Table 3.23: Binary Logistic Regression Analysis of IL-6 A/G Genotypes in the Dominant and Recessive Models.

Genotype IL6	**Study Subjects (n=700)**		**OR (95% CI)**	**p-value**
	Control (n=350) n (%)	**T2DM (n=350) n (%)**		
Dominant Model (AA vs AG+GG)				
AA	230 (66)	173 (49)	1 (Ref)	
AG+GG	120 (34)	177 (51)	1.96 (1.45 – 2.66)	<0.001
Recessive Model (GG vs AA+AG)				
GG	17 (5)	157 (44)	1 (Ref)	
AA+AG	334 (95)	193 (56)	0.06 (0.04– 0.11)	<0.001

Results were expressed as number (percentage); The level of significance was set to $p<0.05$; OR=Odds ratio; CI=Confidence interval; AA= genotype homozygous normal; AG= heterozygous mutant, and GG= homozygous mutant

Table 3.24 showed the frequency distribution of IL-6 A238G genotypes in male participants of the study population. Here only the homozygous mutant (GG) variant demonstrated a significant association ($p<0.001$) with type 2 DMand odds having homozygous mutant variant GG offered 9.39 times more likely to develop T2DM compared to control group homozygous normal AA genotype among the male study population (95%CI=4.29 to 20.56). Moreover, the heterozygous mutant variant (AG) was significantly 85% less likely to develop T2DM than the control group AA genotype (95%CI=0.07- 0.31).

Table 3.24: Distribution of IL-6 (A597G) Genotypes in Male StudySubjects

Genotype	Study Subjects (n=300)		OR (95% CI)	p-value
	Control (n=350) n (%)	T2DM (n=350) n (%)		
AA	97 (57)	93 (54)	1(Ref)	
AG	70 (39)	10 (5)	0.15 (0.07- 0.31)	<0.001
GG	8 (4)	72 (41)	9.39 (4.29- 20.56)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; AA= genotype homozygous normal; AG= heterozygous mutant, and GG= homozygous mutant

Table 3.25 showed the frequency distribution of IL-6 A597G Genotypes in female participants. Homozygous mutant variant (GG) showed a significant (p<0.001) association and was 15.70 times more likely to develop type 2 DM compared to the control homozygous normal AA genotype among the female study population and (OR=15.70, 95% CI=7.48- 32.94). In contrast, a heterozygous mutant variant (AG) was not significantly correlated with type 2 DM.

Table 3.25: Distribution of IL-6 (A597G) Genotypes in Female StudySubjects

Genotype	Study Subjects (n=350)		OR (95% CI)	p-value
	Control (n=175) n (%)	T2DM (n=175) n (%)		
AA	133 (76)	80 (45)	1 (Ref.)	
AG	33 (19)	10 (5)	0.50 (0.24- 1.08)	>0.05
GG	9 (5)	85 (50)	15.70 (7.48-32.94)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; AA= genotype homozygous normal; AG= heterozygous mutant, and GG= homozygous mutant

Table 3.26 showed the association of age differences in female participants with IL-6 A597G polymorphism and their significance with T2DM. It showed a significant (p<0.001) association with homozygous mutant AA genotype in all age range groups of female participants with T2DM. Odds of the homozygous mutant of IL-6 GG genotype were 52.50, 22.04 and15.71 times more likely to develop T2DM compared to the control AA genotype where 95% CI was (6.16-44.77), (7.50-64.77) and (3.33-74.16) respectively in all age groups of female subjects.

Table 3.26: Association of IL-6(A597G) polymorphism with Category of Female Age Group

(year)	Genotype	Study Subjects (n=350)		OR (95% CI)	p-value
		Control (n=175)	T2DM (n=175)		
30-40	**AA**	35	10	1 (Ref.)	
	AG	04	3	2.63 (0.50- 13.72)	>0.05
	GG	01	15	52.50 (6.16- 44.77)	<0.001
41- 50	**AA**	90	49	1 (Ref.)	
	AG	05	4	1.47 (0.38- 5.73)	>0.05
	GG	04	48	22.04 (7.50- 64.77)	<0.001
51- 60	**AA**	30	21	1 (Ref.)	
	AG	04	3	1.07 (0.22- 5.30)	>0.05
	GG	02	22	15.71 (3.33-74.16)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; AA= genotype homozygous normal; AG= heterozygous mutant, and GG= homozygous mutant

Table 3.27 showed the distribution of IL-6 (A597G) genotypes according to the history of hypertension. Here, both homozygous (GG) and heterozygous (AG) mutant variant genotypes showed a significant (p<0.001) association with T2DM. Homozygous mutant variant GG had a significantly higher risk of association with T2DM in both (positive and negative history of HTN) groups where OR, 95%CI were (6.19, 1.38 – 27.82) and (15.16, 8.27 – 27.79) respectively and heterozygous mutant AG genotype showed 85% and 70% less likely to develop T2DM compared to control homozygous wild AA genotype in positive and negative history of Hypertension respectively.

Table 3.27: Distribution of IL-6(A597G) Genotypes according to History of Hypertension

History of Hypertension	Genotype	Study Subjects (n=700)		OR (95% CI)	p-value
		Control (n=350)	T2DM(n=350)		
Yes	AA	16	93	1 (Ref.)	
	AG	12	10	0.14 (0.05-0.39)	<0.001
	GG	2	72	6.19 (1.38- 27.82)	<0.01
No	AA	214	80	1 (Ref.)	
	AG	91	10	0.29 (0.14- 0.59)	<0.001
	GG	15	85	15.16 (8.27-27.79)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; AA= genotype homozygous normal; AG= heterozygous mutant, and GG= homozygous mutant

Table 3.28 showed the distribution of IL-6(A597G) genotypes according to the family history of diabetes and showed significant (p<0.001) association was found for both heterozygous (AG) and homozygous mutant (GG) variants in both (positive or negative family history of DM) groups. Odds of homozygous mutant genotype GG in both (positive and negative family history) groups were10.92 times and 10.44 times more likely to develop T2DM compared to the control homozygous wild AA genotype where 95% CI were (4.84 – 24.66) and (5.14 – 21.18), respectively.

Table 3.28: Distribution of IL-6(A597G) Genotypes According to the Family History of Diabetes

Family History of DM	Genotype	Study Subjects (n=700)		OR (95% CI)	p-value
		Control (n=350)	T2DM (n=350)		
Yes	AA	82	118	1 (Ref.)	
	AG	35	18	0.36 (0.19 – 0.67)	<0.001
	GG	7	110	10.92 (4.84– 24.66)	<0.001
No	AA	147	54	1 (Ref.)	
	AG	67	4	0.16 (0.06– 0.47)	<0.001
	GG	12	46	10.44 (5.14– 21.18)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; AA= genotype homozygous normal; AG= heterozygous mutant, and GG= homozygous mutant

Table 3.29 showed the distribution of IL-6(A597G) genotypes according to their smoking habit and showed a significant (p<0.001) risk of association with heterozygous mutant AG genotype in both smoker and non-smoker groups. Odds of heterozygous mutant AG genotype were 95% and 62% less likely to develop T2DM compared to the control homozygous wild AA genotype in smokers and non-smoker, respectively.

Table 3.29: Distribution of IL-6(A597G) Genotypes according to their smoking habit

History of Smoking	Genotype	Study Subjects (n=700)		OR (95% CI)	p-value
		Control (n=350)	T2DM n=350		
Smoker	AA	10	16	1 (Ref.)	
	AG	29	2	0.04 (0.08 – 0.22)	<0.001
	GG	4	9	1.40 (0.34– 5.81)	>0.05
Non-Smoker	AA	220	157	1 (Ref.)	
	AG	74	20	0.37 (0.22- 0.64)	<0.001
	GG	13	146	15.74(8.60-28.77)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; AA= genotype homozygous normal; AG= heterozygous mutant, and GG= homozygous mutant

Table3.30: showed the distribution of IL-6 (A597G) genotypes according to the category of BMI and showed a significant ($p<0.001$) association which was found for the homozygous mutant variant (GG) in all categories of BMI. Odds of homozygous mutant variant GG in all three groups were 8.074 times, 22.62 times, and 20.57 times more likely to develop T2DM compared to the control homozygous wild AA genotype where OR, 95% CI were (4.05– 16.09), (7.94–64.41), and (2.52– 167.90) respectively.

Table3.30: Distribution of IL-6 (A597G) Genotypes According to the Category of BMI

Category of BMI	Genotype	Study Subjects (n=700)		OR (95% CI)	p-value
		Control (n=350)	T2DM (n=350)		
Normal	AA	108	68	1 (Ref.)	
	AG	51	7	0.21(0.09– 0.50)	<0.001
	GG	12	61	8.07 (4.05– 16.09)	<0.001
Overweight	AA	95	84	1 (Ref.)	
	AG	42	11	0.29 (0.14– 0.61)	<0.001
	GG	4	80	22.62 (7.94– 64.41)	<0.001
Obese	AA	27	21	1 (Ref.)	
	AG	10	2	0.25 (0.05– 1.30)	>0.05
	GG	1	16	20.57 (2.52– 167.90)	<0.001

Results were expressed as number (percentage); The level of significance was set to $p<0.05$; OR=Odds ratio; CI=Confidence interval; AA= genotype homozygous normal; AG= heterozygous mutant, and GG= homozygous mutant

Table 3.31 showed the distribution of IL-6(A597G) genotypes according to HBA_1C status and showed significant ($p<0.05$) association with T2DM for heterozygous mutant (AG) and homozygous mutant (GG) in HBA_1C <6.5 group. Odds of heterozygous mutant (AG) and homozygous mutant (GG) in the HBA_1C <6.5 group were 2.02 times and 15.29 times more likely to develop T2DM compared to the control homozygous wild AA genotype.

Table 3.31: Distribution of IL-6 (A597G) Genotypes According to HBA$_1$C Status

HBA$_1$C	Genotype	Study Subjects (700)		OR (95% CI)	p-value
		Control (n=350)	T2DM (n=350)		
≤6.5%	AA	225	45	1 (Ref.)	
	AG	99	40	2.02 (1.24–3.28)	<0.01
	GG	17	52	15.29 (8.11–28.84)	<0.001
≥6.6%	AA	4	58	1 (Ref.)	
	AG	3	65	1.49 (0.32 –6.96)	>0.05
	GG	2	90	3.10 (0.55–17.50)	>0.05

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; AA= genotype homozygous normal; AG= heterozygous mutant, and GG= homozygous mutant

Table 3.32 showed the glycemic, insulinemic status according to IL-6 A597G genotype in both T2DM and control. It showed no significant distribution with fasting blood glucose, 2 hours after breakfast, HBA$_1$C%, serum insulin, HOMA-IR, HOMA B%, Secret-HOMA in control, and a significant distribution were found with HOMA B% and Secret HOMA in type 2 diabetic subjects, and they were (137.00±11.01 for AA;293±443.02 for AG;112±7.03 for GG and 145.04±11.07 for AA;322.03±13.07 for AG;108.04±21.05 for GG) respectively.

Table 3.32: Study of Glycemic and Insulinemic Biomarkers According to IL-6 (A597G) Genotypes

Variables	Study Subjects (700)					
	T2DM (n=350) Genotype			Control (n=350) Genotype		
	AA (n=173)	AG (n=26)	GG (n=151)	AA (n=232)	AG (n=14)	GG (n=104)
FBS (mmol/L)	8.50±2.95	8.62±2.67	9.08±3.00	4.80±1.31	5.05±1.18	4.82±0.97
ABF (mmol/L)	11.78±4.05	11.81±3.53	12.56±4.12	6.86±1.17	6.97±1.48	7.07±1.50
HbA$_1$C (%)	7.22±1.71	6.95± 1.90	7.34±1.80	5.20±0.77	5.11±0.73	5.32±0.65
Insulin(µU/L)	22.68±16.15	28.54±21.60	23.14±14.30	16.36±11.13	19.20±15.90	14.90±90.70
HOMA IR	8.80±7.93	11.04±10.80	9.83±6.81	3.46.00±2.73	4.37±4.91	3.08±1.96
HOMA B%	137.00±11.01	293±443.02	112±7.03**	275.03±58.02	252.04±174.02	392.03±87.01
Secrt-HOMA	145.04±11.07	322.03±13.07	108.04±21.05*	318.01±67.03	291.04±201.03	452.05±100.04

Results were presented as mean ± standard deviation (SD). Differences were considered significant at p<0.05;*=p<0.01; **=P<0.001 HOMA B%= expressed Beta Cell Function; HOMA S%= expressed Beta Cell Sensitivity; HOMA-IR: expressed Homeostasis Model of Assessment - Insulin Resistance; Secretory HOMA: Secretory Homeostasis Model of Assessment.

Figure 3.9 showed a comparison of HOMA-IR level among AA, AG, and GG variant in T2DM subjects by box plot diagram. It showed positive skewness of data in homozygous normal AA genotype and homozygous mutant variant GG genotype. As shown in figure 3.9, the home-IR level in the diabetes group was not significantly ($p>0.05$) different among AA, AG, and GG variant.

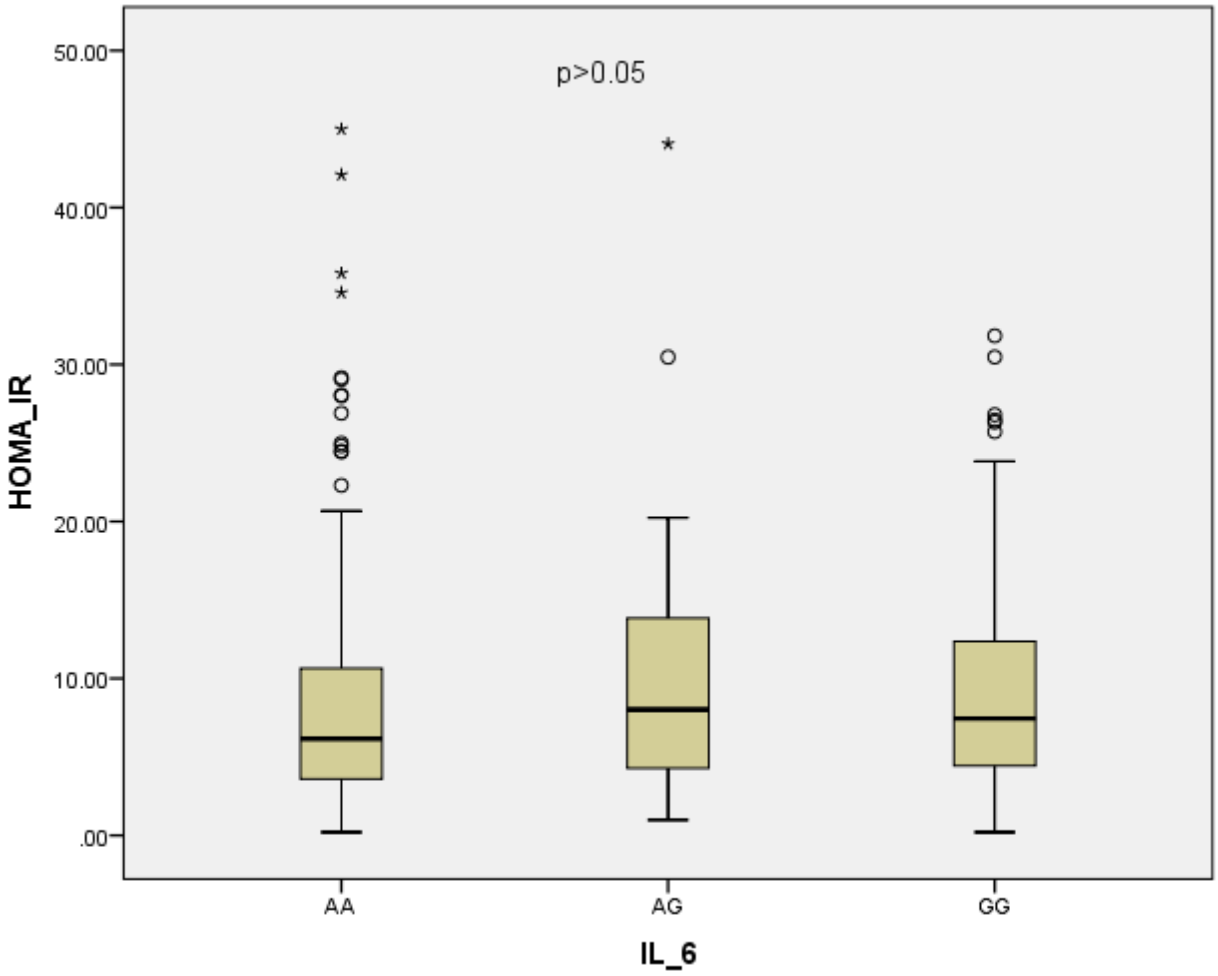

Figure 3.9:Comparison of HOMA-IR level among AA, AG, and GG variant in T2DM Subjects

Figure 3.10 showed a comparison of serum insulin level among AA, AG, and GG variants in T2DM subjects. This box plot showed positive skewness of data in homozygous normal AA genotype and homozygous mutant variant GG genotype. As shown in figure 3.10, the serum insulin level in the diabetes group was not significantly ($p>0.05$) different among AA, AG, and GG variant.

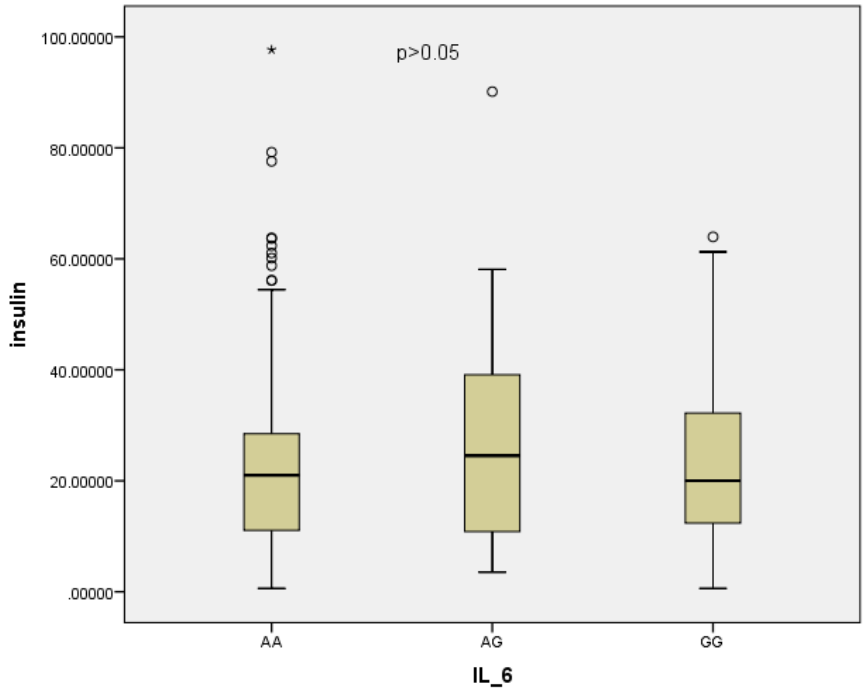

Figure 3.10:Comparison of Serum Insulin level among AA, AG, and GG variant in T2DM Subjects

Table 3.33 showed the lipidemic status according to IL-6 A597G genotype in both T2DM and control and showed the significant distribution was with HDL-C only in the control group, and it was (45.34±17.57 for AA;56.71±19.53 for AG;44.98±15.39 for GG), whereas in type 2 diabetic subjects showed no considerable distribution with serum cholesterol, triacylglycerol, high-density lipoprotein, low-density lipoprotein, and visceral adiposity index.

Table 3.33: Study of Lipidemic Biomarkers According to IL-6 (A597G) Genotypes

Variables	Study Subjects (n=700)					
	T2DM (n=350) Genotypes			Control (n=350) Genotypes		
	AA (n=173)	AG (n=20)	GG (n=157)	AA (n=232)	AG (n=14)	GG (n=104)
Cholesterol(mg/dL)	185.75±41.67	164.85±34.26	186.56±44.70	174.74±103.00	165.66±52.64	193.65±129.56
Triaglyceride (mg/dL)	188.17±94.83	168.93±8.61	193.47±120.03	136.87±77.82	152.57±79.95	153.96±105.84
HDL-C (mg/dL)	37.74±7.17	37.30±6.86	38.83±7.60	45.34±17.57	56.71±19.53	44.98±15.39 *
LDL-C (mg/dL)	114.89±42.66	97.05±36.92	113.89±41.75	101.01±69.43	95.72±42.01	116.43±100.51
VAI	3.86±2.43	3.53±2.16	4.66±3.07	2.58±2.12	2.29±1.38	2.68±2.52

Results were presented as mean ± standard deviation (SD). Groups of data were compared using a one-way ANOVA test. The p-value was<0.05 taken as the significance level; HDL-C=High Density Lipoprotein; LDL-C=Low Density Lipoprotein; VAI= Visceral Adiposity Index; *=p<0.05; TC= Total cholesterol; TG=triglyceride; HDL-C=high density lipoprotein cholesterol; LDL-C=low density lipoprotein cholesterol;VAI=visceral adiposity index

Table 3.34 showed the association of IL6 A597G polymorphism with glycemic parameters in the study population and showed different polymorphic (AA, AG, GG) variants of IL6 A597G polymorphism were significantly ($p<0.05$) correlated with fasting blood glucose, 2hours after breakfast, glycated hemoglobin (HBA_1C), insulin, the homeostatic model of insulin resistance (HOMA-IR) and were non significantly correlated with HOMA B% and Secret-HOMA.

Table 3.34: Association of IL6 A597G Polymorphism with Glycemic Parameters in the Study Subjects

Variables	Genotype	Genotype	F-value	p-value
FBS[a,d] (mmo/L)	AA	AG*		
		GG**		
	AG	AA*		
		GG**	45.36	<0.001
ABF[a,d] (mmol/L)	AA	AG*		
		GG**	49.26	<0.001
	AG	AA*		
		GG		
HBA_1C[a,d] (%)	AA	AG**		
		GG**		
	AG	AA**		
		GG**	42.81	<0.001
HOMA-IR[a,d]	AA	AG		
		GG**		
	AG	AA		
		GG**	17.43	<0.001
HOMA B%[a,d]	AA	AG		
		GG		
	AG	AA	2.36	>0.05
		GG		
Secret HOMA[a,d]	AA	AG		
		GG		
	AG	AA	2.533	>0.05
		GG		
insulin [b,c] (µU/L)	AA	AG		
	GG	GG		
		AA*		
		AG*	5.39	<0.05

Here, the a=Welch ANOVA test, since data violated homogeneity, b=One -way ANOVA Test, since data confirmed homogeneity, and c=LSD test, since data-approved d=Dunnett test; since data violated homogeneity. The change was seen as significant at $p<0.05$; *= POST HOC $p<0.05$; **= POST HOC $p<0.001$

Table 3.35 showed the association of IL-6 A597G polymorphism with lipidemic and clinical parameters in the study population. It showed polymorphic (AA, AG, GG) variants of IL-6 A597G polymorphism were significantly($p<0.05$) correlated with serum cholesterol, HDL-C, visceral adiposity index, waist-hip ratio, and systolic blood pressure and were non significantly correlated with age, body mass index, triacylglycerol, diastolic blood pressure, and low-density lipoprotein.

Table 3.35: Association of IL6 A597G polymorphism with Lipidemic and Clinical Parameters in the Study Subjects

Variables	Genotype	Genotype	F- Value	p-value
BMI [a,d] **(Kg/m2)**	AA	AG		
		GG		
	AG	AA	0.51	>0.05
		GG		
Cholesterol[a,d] **(mg/dL)**	AA	AG*		
		GG*	7.89	<0.01
	AG	AA*		
		GG		
HDL- C[a,d] **(mg/dL)**	AA	AG*		
		GG*		
	AG	AA*	15.58	<0.001
		GG**		
LDL-C[a,d] **(mg/dL)**	AA	AG		
		GG	3.61	>0.05
	AG	AA		
		GG		
VAI [a,d]	AA	AG**		
		GG		
	AG	AA**	15.55	<0.001
		GG**		
AGE[b,c] **(Year)**	AA	AG		
		GG		
	AG	AA		
		GG*	3.02	<0.05
WHR[b,c]	AA	AG*		
		GG**	7.50	<0.001
	AG	AA*		
		GG		
SBP [b, c] **(mm Hg)**	AA	AG		
		GG**		
	AG	AA		
		GG**	12.54	<001
DBP[b,c] **(mm Hg)**	AA	AG		
		GG*	4.50	<0.01
	AG	AA		
		GG		
TG[b,c] **(mg/dL)**	AA	AG		
		GG**	8.55	<0.001
	AG	AA		
		GG		

Here, the a=Welch ANOVA test, since data violated homogeneity, b=One -way ANOVA Test, since data confirmed homogeneity, and c=LSD test, since data confirmed homogeneity, d=Dunnett Test since data violated homogeneity. Changes were seen to be significant at p<0.05; *= POST HOC p<0.05; **= POST HOC p <0.001;TC= Total cholesterol; TG=triglyceride; HDL-C=high density lipoprotein cholesterol; LDL-C=low density lipoprotein cholesterol; VAI=visceral adiposity index

INTERLEUKIN- TEN (IL-10) GENE ANALYSIS

(rs no 1800872)

3.6 Determination of IL-10 Gene Genotype

Figure 3.11 showed the 241 base pair PCR product of the IL-10 gene after visualisation on 2% agarose gel.

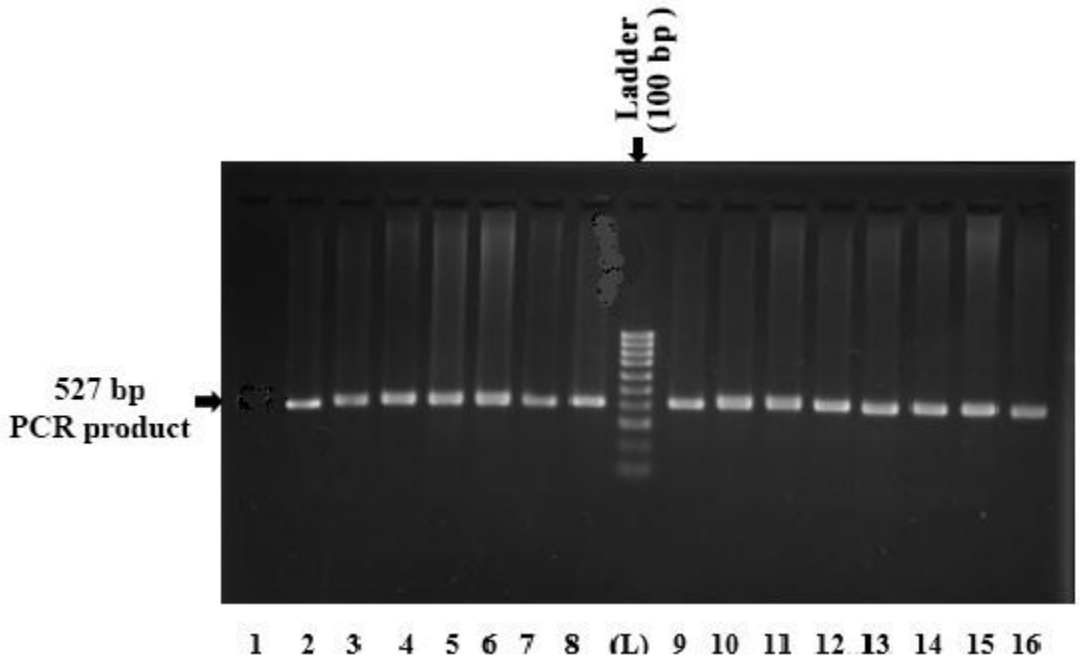

Figure 3.11:Agarose gel image of PCR product of IL10 gene

3.6.1 RFLP analysis of the IL10 gene candidate marker

In the present study, IL-10 592C>A was used as a candidate marker. The Rsa*1* restriction enzyme analyzed polymorphism.

Rsa*1* Restriction digestion: 241bpPCR products were digested for 1 to 4 hours with *Rsa1* at 37°C. RFLP products were directly separated by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining. *Rsa1* digestion produced three fragments depending on the genotype; Heterozygous (Ht) variant genotype CA: 241bp,166bp, and 75bp; Homozygous (Hz)variant genotype AA: 241bp; Homozygous (Hz) wild genotype CC: 166bp and75bp. Figure 3.12 presented the digestion pattern of the IL-10 gene. Here restriction digestion produced following fragments: CA variant: 241bp and 166 bp found in lane 1,4,6; AA variant:241bp found in lane 3, 5,8; CC variant: 166 bp found in lane 2, 7.

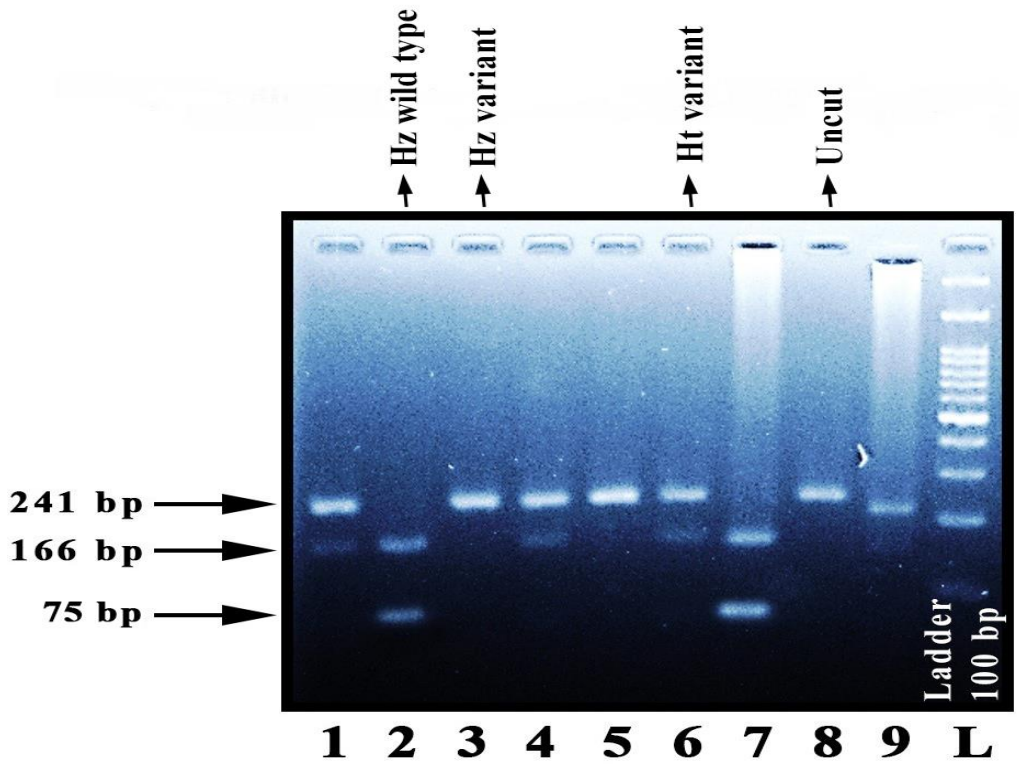

Figure 3.12: Agarose gel image of Rsa1 digested PCR product of 1L-10 gene.

Table 3.36 demonstrated that IL-10 C592A genotypic frequencies follow the Hardy-Weinberg Equilibrium in the control group.

Table 3.36: Hardy-Weinberg Equilibrium for Comparison of Genotype Frequencies in T2DM and Control for IL10 gene

Hardy Online Calculator	**Study Subjects (n=700)**					
	T2DM (n= 350)			**Control** (n=**350)**		
	IL-10 (C592A) **Genotype**			IL-10 (C592A) **Genotype**		
	CC	**CA**	**AA**	**CC**	**CA**	**AA**
Observed value	103	105	142	220	110	20
Expected Value	69	173	108	216	117	16
Chi-square value		54			1.5	

Table 3.37 showed the genotype distributions and allele frequency of IL-10(C592A) polymorphism. The percentages of CC, CA, and AA genotypes at 592 positions of IL-10 gene were 63%, 32%, and 5%, respectively, in the control group, while in the T2DM group, it was 29%, 30%, and 41%, respectively. Both the homozygous mutant variant (AA) and heterozygous mutant (CA) of the IL10 genotype showed a significant($p<0.001$) association with T2DM. Odds having heterozygous mutant CA and homozygous mutant AA were 2.04 times and 15.17 times more likely to develop T2DM compared to the control CC genotype (95%CI=1.43 to 2.91 and 8.98 to 25.60), respectively. Minor allele frequency was significantly ($p<0.001$) higher in T2DM than in control.

Table 3.37: Genotype Distributions and Allele Frequency of IL 10(C592A) Polymorphism

Genotype	Study Subjects (n=700)		OR (95% CI)	p-value
	Control Group (n=350) n (%)	Diabetic Group (n=350) n (%)		
CC	220(63)	103(29)	1(Ref)	
CA	110(32)	105(30)	2.04 (1.43 -2.91)	<0.001
AA	20(5)	142(41)	15.17 (8.98 - 25.60)	<0.001
Allele	**(n, frequency)**			
C	550(79)	311(44)		
A	150(21)	389(56)	4.58 (3.62 -5.79)	<0.001

Results were expressed as number (percentage); The level of significance was set to $p<0.05$; OR=Odds ratio; CI=Confidence interval; CC= genotype homozygous normal; CA= heterozygous mutant, and AA= homozygous mutant.

Figure 3.13 showed the distribution of IL-10 (C592A) genotype in the study population. This Bar diagram showed that homozygous wild genotype CC carrying individuals in T2DM and control group were 103 and 220, respectively. Heterozygous mutant variant CA genotype carrying individuals in T2DM were 105 and 110 in the control group, whereas homozygous mutant variant genotype AA carrying individuals was 142 in T2DM and 20 in the control group.

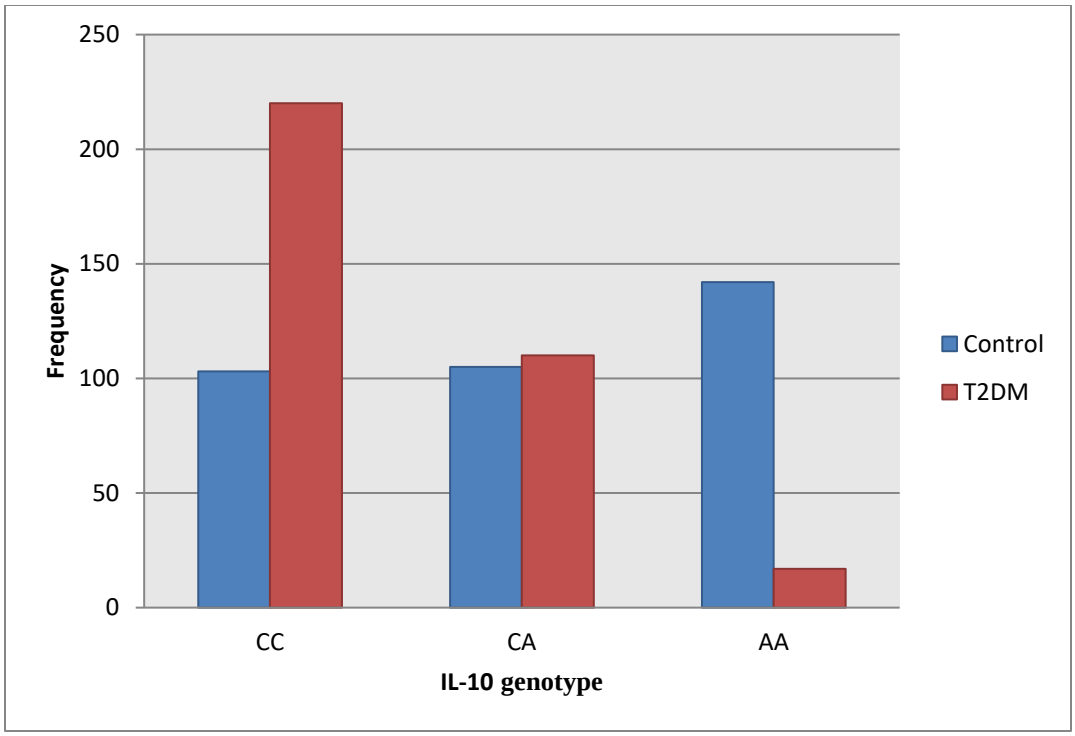

Figure 3.13: Distribution of IL-10 C592A Genotypes in the study population (n=700)

Table 3.38 showed the binary logistic regression analysis of IL-10 C592A genotypes in the dominant (CC versus CA+AA) and the recessive model (AA versus CC+CA). Here, CC versus the combination of CA and AA genotype was considered as the dominant model, and AA versus the combination of CC genotype and CA genotype were considered as recessive models collectively. It was also found that in the dominant model, the frequency of CA+AA genotype was significantly ($p<0.001$)correlated with T2DM, and the odds was 4.058 times more likely to develop T2DM in the cases of having CA or AA genotype compared to CC genotype where 95%CI was (2.95-5.56). It was found that in the recessive model, the frequency of the CC+CA genotype also had a significant ($p<0.001$)association with T2DM; the odds was 92% times less likely to develop T2DM in the cases of having CC or CA genotype compare to AA genotype.

Table 3.38: Binary Logistic Regression Analysis of IL-10 C592A Genotypes in Dominant and Recessive model

Genotype	Study Subjects (n=700)		OR (95% CI)	p-value
	Control (n=350)	T2DM (n=350)		
Dominant Model (CC vs AA)				
CC	220(63)	103(29)	**1 (Ref)**	
CA+AA	130 (37)	247 (71)	4.06 (2.95-5.56)	<0.001
Recessive Model (AA vs CC+CA)				
AA	20 (6)	142 (40)	**1 (Ref)**	
CC+CA	330 (94)	208 (60)	0.09 (0.05-0.14)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; CC= genotype homozygous normal; CA= heterozygous mutant, and AA= homozygous mutant

Table 3.39 showed the frequency distribution of IL-10 C592A genotypes in male participants and the risk of diabetes and showed both heterozygous mutant variant CA and homozygous mutant variant AA significant(p<0.001) association with T2DM. Odds of heterozygous mutant genotype CA and homozygous mutant genotype AA were 1.73 times and 17.47 times more likely to develop T2DM than control homozygous wild CC genotype where 95 % CI were(1.03 - 2.89), (8.34-36.59) respectively in the male study subjects.

Table 3.39: Distribution of IL-10(C592A) Genotypes in Male StudySubjects

Genotype	Study subjects (n=350)		OR (95% CI)	p-value
	Control (n=175) n (%)	T2DM (n=175) n (%)		
CC	107 (61)	49 (28)	1 (Ref.)	
CA	58 (34)	46 (26)	1.73(1.03- 2.89)	<0.05
AA	10 (5)	80 (46)	17.47(8.34-36.59)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; CC= genotype homozygous normal; CA= heterozygous mutant, and AA= homozygous mutant

Table 3.40 showed frequency distribution of IL-10 C592A genotypes in female participants and risk of diabetes and showed both heterozygous (CA), and homozygous mutant (AA)variant was significantly (p<0.001)correlated with type-2DM and Odds of heterozygous mutant CA and homozygous mutant CC genotype were2.37 and 12.97 times more likely to develop T2DM

compared to control homozygous wild CC genotype and 95% CI were (1.44- 3.89), (6.17–27.26) respectively.

Table 3.40: Distribution of IL-10(C592A) Genotypes in Female StudySubjects

Genotype	Study Subjects (n=350)		OR (95% CI)	p-value
	Control(n= 175) n (%)	T2DM(n= 175) n (%)		
CC	113 (65)	54 (30)	1 (Ref.)	
CA	52 (30)	59 (34)	2.37 (1.44 -3.89)	<0.001
AA	10 (5)	62 (36)	12.97 (6.17-27.26)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; CC= genotype homozygous normal; CA= heterozygous mutant, and AA= homozygous mutant

Table 3.41 showed the association of age differences in female participants with IL-10 C592A polymorphism. Their significance with T2DM showed a significant(p<0.001)association with T2DM with all age range groups of female participants. This table also showed odds of both homozygous mutant AA and heterozygous mutant CA genotypes of all age range groups were 20.81 times, 50.88 times, 35.78 times, 27.60 times, 15.47 times, and 30.94times more likely to develop T2DM compared to control homozygous wild CC genotype where 95%CI were (3.75–115.4), (5.719–452.6), (10.26–124.8), (9.07–83.95), (3.14–76.01) and (3.74–255.4) respectively.

Table 3.41: Association of IL-6(A597G) Polymorphism with Category of Female Age Group

(Year)	Genotype	Study Subjects (n=350)		OR (95% CI)	p-value
		Control (n=175)	T2DM (n=175)		
30-40	CC	37	8	1 (Ref)	
	CA	2	9	20.81 (3.75–115.4)	<0.001
	AA	1	11	50.88 (5.71–452.6)	<0.001
41- 50	CC	92	30	1 (Ref)	
	CA	03	35	35.786 (10.26–12.8)	<0.001
	AA	04	36	27.60 (9.07–83.95)	<0.001
51- 60	CC	33	16	1 (Ref)	
	CA	02	15	15.47 (3.14–76.01)	<0.001
	AA	01	15	30.94 (3.74–255.4)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; CC= genotype homozygous normal; CA= heterozygous mutant, and AA= homozygous mutant

Table 3.42 showed the distribution of IL-10(C592A) genotypes according to the history of hypertension and showed significant (p<0.05) association with both heterozygous mutant CA and homozygous mutant genotype AA among participants having a negative history of hypertension, whereas only homozygous mutant genotype AA showed significant association with T2DM among participants having a positive history of hypertension. Odds having homozygous mutant variant AA genotypes were 10.73 times and 16.16 times more likely to develop T2DM compared to control homozygous wild CC in both positive hypertensive and negative hypertensive groups, respectively.

Table 3.42: Distribution of IL-10(C592A) Genotypes According to History of Hypertension

History of Hypertension	Genotype	Study Subjects (n=700)		OR (95% CI)	p-value
		Control (n=350)	T2DM(n=350)		
Yes	CC	16	41	1 (Ref.)	
	CA	12	41	1.33 (0.56–3.16)	>0.05
	AA	2	55	10.73 (2.33–49.31)	<0.001
No	CC	204	61	1 (Ref.)	
	CA	98	65	2.21 (1.54–3.39)	<0.001
	AA	18	87	16.16 (9.02–28.95)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; CC= genotype homozygous normal; CA= heterozygous mutant, and AA= homozygous mutant

Table 3.43 according to the family history of diabetes, IL-10 C592A genotypic risk of DM showed significant (p<0.001) association with T2DM for both heterozygous mutant CA and homozygous mutant genotype AA among participants having a positive family history of diabetes. At the same time, only homozygous mutant genotype AA had shown a significant (p<0.001) association with T2DM among participants having a negative family history of diabetes. Odds of both heterozygous mutant CA and homozygous mutant genotype AA were2.45 times and 16.06 times more likely to develop T2DM than control homozygous wild CC in a positive family history of diabetes, respectively. Odds having homozygous mutant variant AA genotype was10.96 times more likely to develop T2DM in a negative family history of diabetes.

Table 3.43: Distribution of IL-10 (C52A) Genotypes According to Family History of DM

Family History of DM	Genotype	Study Subjects (n=700)		OR (95% CI)	p-value
		Control(n=350)	T2DM (n=350)		
Yes	CC	80	66	1 (Ref.)	
	CA	36	73	2.45 (1.46– 4.11)	<0.001
	AA	8	106	16.06 (7.29– 35.36)	<0.001
No	CC	139	37	1 (Ref.)	
	CA	75	33	1.65 (0.95-2.857)	>0.05
	AA	12	35	10.96 (5.17–23.18)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; CC= genotype homozygous normal; CA= heterozygous mutant, and AA= homozygous mutant

Table 3.44, according to the smoking habit, both homozygous mutant (AA) and heterozygous mutant genotype (CA) of IL 10 C592A showed a significant (p<0.005) association with T2DM in the non-smoker group. Odds of both homozygous mutant (AA) and heterozygous mutant genotype (CA) were 2.63 times and 19.56 times more likely to develop T2DM than control homozygous wild CC genotype in non -smoker group.

Table 3.44: Distribution of IL-10 (C592A) Genotypes According to Smoking Habit

History of Smoking	Genotype	Study Subjects (700)		OR (95% CI)	p-value
		Control (n=350)	T2DM (n=350)		
Smoker	CC	7	5	1 (Ref.)	
	CA	30	8	0.37 (0.09 - 1.49)	>0.05
	AA	6	16	3.73 (0.84– 16.45)	>0.05
Non-Smoker	CC	213	98	1 (Ref.)	
	CA	80	97	2.63 (1.80–3.85)	<0.001
	AA	14	126	19.56 (10.71-35.71)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; CC= genotype homozygous normal; CA= heterozygous mutant, and AA= homozygous mutant

Table 3.45, according to the category of BMI, both homozygous mutant (AA) and heterozygous mutant genotype (CA) of IL-10 C592A showed a significant (p-<0.05) association with T2DM among normal, overweight, and obese BMI group. Odds having both homozygous mutant (AA) and heterozygous mutant genotype (CA) were 1.83 times, 28.08 times, 3.54 times, 15.36 times more likely to develop T2DM compared to control homozygous wild CC genotype in both overweight and obese groups were 95%CI were (1.078–3.136), (10.66–10.30), (1.220–10.30) and (2.958 – 79.81) respectively.

Table 3.45: Distribution of IL-10 (C592A) Genotypes According to Category of BMI

Category of BMI	IL10C/A genotype	Study Subjects (700)		OR(95% CI)	p-value
		Control (n=350)	T2DM (n=350)		
Normal	CC	104	42	1 (Ref.)	
	CA	54	43	1.97 (1.15– 3.37)	<0.05
	AA	13	51	9.71 (4.79–19.69)	<0.001
Overweight	CC	90	50	1 (Ref.)	
	CA	46	47	1.83 (1.07–3.13)	<0.05
	AA	5	78	28.08 (10.66–10.30)	<0.001
Obese	CC	26	11	1 (Ref.)	
	CA	10	15	3.54 (1.22–10.30)	<0.05
	AA	2	13	15.36 (2.95 – 79.81)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; CC= genotype homozygous normal; CA= heterozygous mutant, and AA= homozygous mutant

Table 3.46 according to HBA_1C Status, IL-10 C592A genotypic risk of DM showed a significant (p-<0.005) association with T2DM for heterozygous mutant CA genotype in both groups. Odds with homozygous mutant variant AA was 8.80 times more likely to develop T2DM than control homozygous wild CC genotype in HBA_1C<6.5% group.

Table 3.46: Distribution of IL-10(C592A) Genotypes According to HBA_1C Status

HBA_1C Status	IL10C/A Genotype	Study Subjects (n=700)		OR (95% CI)	p-value
		Control (n=350)	T2DM (n=350)		
≤ 6.5%	CC	217	69	1 (Ref.)	
	CA	104	12	0.36 (0.18–0.69)	<0.001
	AA	20	56	8.80 (4.94–15.70)	<0.001
≥6.6%	CC	3	104	1 (Ref.)	
	CA	5	8	0.04 (0.01–0.22)	<0.001
	AA	1	101	2.91 (0.29–28.49)	>0.05

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval; CC= genotype homozygous normal; CA= heterozygous mutant, and AA= homozygous mutant

Table 3.47showed the glycemic, insulinemic status according to IL-10 C592A genotype in both case and control subjects and showed significant (p<0.05) association in FBS level in the control group where mean FBS in homozygous wild CC, heterozygous mutant variant CA, and

homozygous mutant variant AA were (4.95 ±1.26, 4.45 ±1.02 and 4.72 ±1.17) mmol/L respectively. Two hours after breakfast, HbA_1c%, insulin, HOMA-IR, HOMAB%, and secretory HOMA were non-significantly distributed with all genotypes (CC, CA, AA) of IL10 C592A polymorphism in both T2DM and control group.

Table 3.47: Study of Glycemic and Insulinemic Biomarkers According to IL-10(C592A) Genotypes

Variables	Study Subjects (700)					
	T2DM (n=350)			Control (n=350)		
	IL-10 Genotype			IL-10 Genotype		
	CC (n=103)	AC (n=105)	AA (n=142)	CC (n=200)	AC (n=47)	AA (n=103)
FBS (mmol/L)	8.70 ±2.27	8.92 ±3.19	8.70 ±2.90	4.95 ±1.26	4.45 ±1.02	4.72 ±1.17 *
ABF (mmol/L)	11.94 ±3.98	12.42 ±4.17	12.07 ±4.02	6.98 ±1.26	7.03 ±0.97	6.78 ±1.20
HbA_1C (%)	7.09 ±1.74	7.36 ±1.81	7.30 ±1.71	5.22 ±0.72	5.26 ±0.68	5.25 ±0.79
Insulin (µ U/L)	24.13±15.79	24.64±18.23	21.51±13.48	16.32±10.54	15.46±9.58	15.74±11.83
HOMA IR	9.73 ±7.89	9.69 ±8.67	8.43 ±6.50	3.53 ±2.63	2.97 ±2.69	3.29 ±2.80
HOMA B%	125.03±11.02	50.01±85.03	121.04±10.02	386.02±64.05	57.01±85.04	273.02±113.03
SecrtHOMA	139.01±13.04	47.03±98.05	116.02±24.21	446.00±74.11	66.04±131.21	315.04±98.12

Results were expressed as number (percentage); The level of significance was set to $p<0.05$; *=$p<0.05$; OR=Odds ratio; CI=Confidence interval; CC= genotype homozygous normal; CA= heterozygous mutant, and AA= homozygous mutant

Figure 3.14 showed a comparison of HOMA-IR level among CC, CA, and AA variant in T2DM subjects. This box plot showed positive skewness of data in homozygous normal CC genotype and homozygous mutant AA genotype. As shown in figure 3.14, the HOMA-IR level in the diabetes group was not significantly ($p>0.05$) different among CC. CA and AA variant.

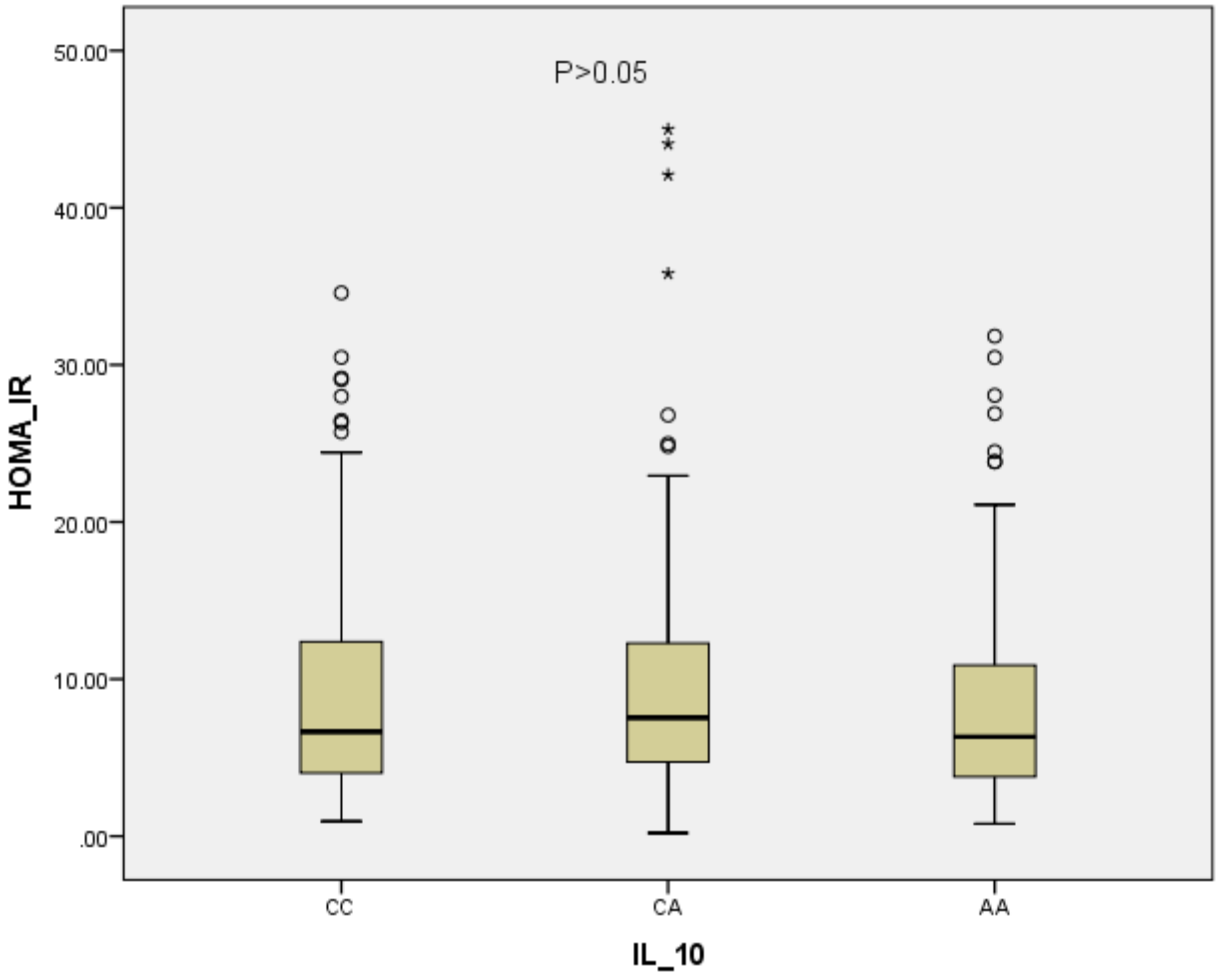

Figure 3.14:Comparison of HOMA-IR level among CC, CA, and AA variant in T2DM Subjects

Figure 3.15 showed a comparison of serum insulin level among AA, AG, and GG variant in T2DM Subjects. This box plot showed positive skewness of data in homozygous normal CC genotype and homozygous mutant variant AA genotype. As shown in figure 3.15, the serum insulin level in the diabetes group was not significantly ($p>0.05$) different among CC. CA and AA variant.

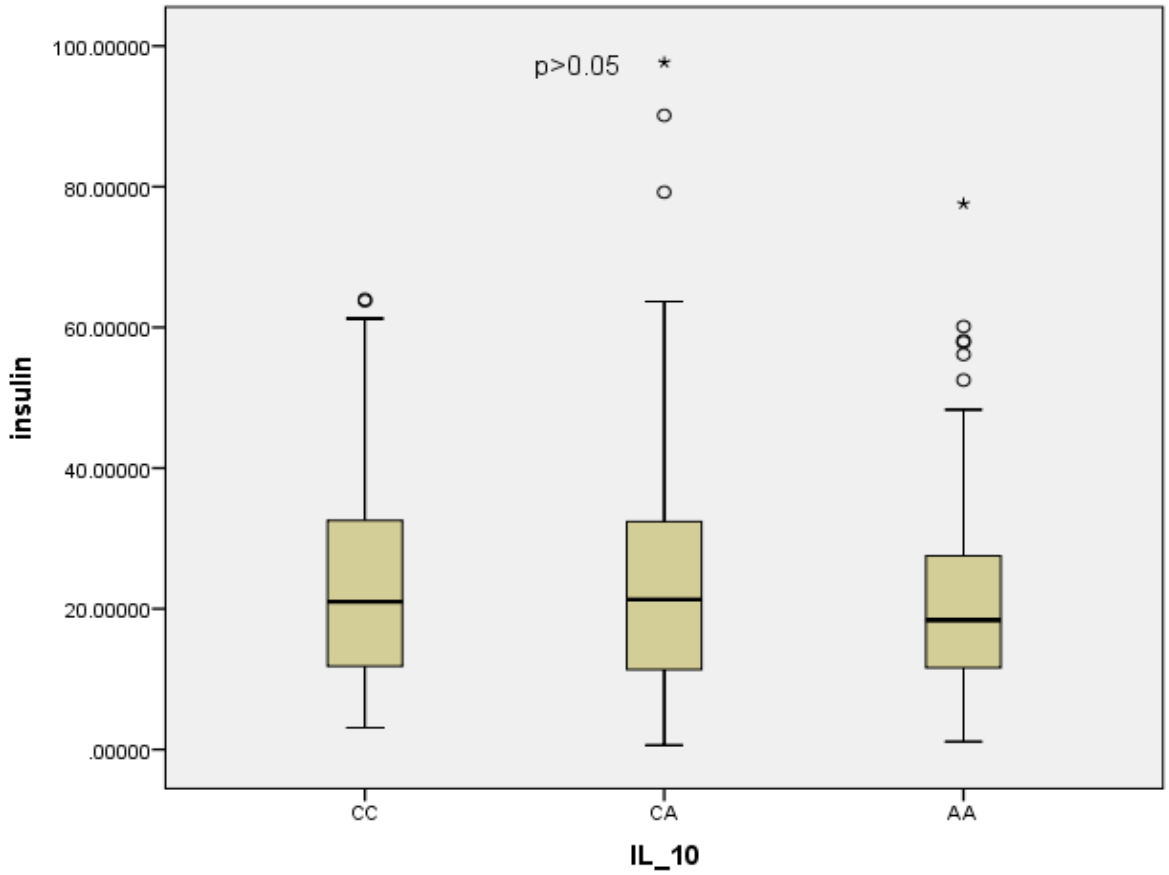

Figure 3.15:Comparison of Serum Insulin level among AA, AG, and GG variant in T2DM Subjects

Table 3.48 showed the lipidemic status according to IL-10 C592A genotype in both T2DM and control subjects. It showed a significant ($p<0.01$)association with serum high-density cholesterol levels in both control and T2DM, and mean serum high-density cholesterol level in homozygous wild CC, heterozygous mutant CA and homozygous mutant AA were (45.43±17.19mg/dL, 38.67±16.59mg/dL, and 95.44±77.96 mg/dL) and (39.82±7.54mg/dL,38.50±7.63mg/dL and 36.81±6.73mg/dL), respectively. Low-density cholesterol (LDL-C) was significantly ($p<0.01$) distributed in control group and these were (105.76±77.79 mg/dL for AA,125.55±86.23 mg/dL for AC and 95.38±77.31 mg/dL for CC) respectively .Total cholesterol, triglyceride, and visceral adiposity index were non-significantly correlated with all genotypes (CC, CA, AA) of IL10-C592A polymorphism in both the T2DM and control group.

Table 3.48: Study of Lipidemic Biomarkers According to IL-10 (C592A) Genotypes

	Study Subjects (n=700)					
	T2DM (n=350) Genotype			Control(n=350) Genotype		
Variables	**AA (n=103)**	**AC (n=105)**	**CC (n=142)**	**AA (n=200)**	**AC (n=47)**	**CC (n=103)**
TC (mg/dL)	182.98±42.16	192.65±97.47	188.02±42.26	181.08±107.03	186.54±86.9	174.49±125.19
TG (mg/dL)	173.51±89.25	182.65±44.48	198.64±122.21	151.06±95.69	128.00±93.49	132.74±62.61
HDLC(mg/dL)	39.82±7.54	38.50±7.63	36.81±6.73 *	45.43±17.19	38.67±16.59	95.44±77.96 *
LDL-C (mg/dL)	112.96±42.78	106.94±37.34	118.54±44.30	105.76±77.79	125.55±86.23	95.38±77.31 *
VAI	3.58±2.40	4.01±2.47	4.12±3.08	2.78±2.31	2.78±2.74	2.16±1.68

Results were expressed as number (percentage);The level of significance was set to $p<0.05$; *=$p<0.01$; OR=Odds ratio; CI=Confidence interval;TC= Total cholesterol; TG=triglyceride; HDL-C=high density lipoprotein cholesterol; LDL-C=low density lipoprotein cholesterol;VAI=visceral adiposity index ;CC= genotype homozygous normal; CA= heterozygous mutant, and AA= homozygous mutant

Table 3.49 showed the association of IL-10 C592A polymorphism with glycemic and some clinical parameters in the study population. It showed polymorphic (CC, CA, AA) variants of IL-10C592A polymorphism had a significant association with fasting blood glucose, 2hours after breakfast, glycated hemoglobin (HBA_1C), fasting insulin, the homeostatic model of insulin resistance (HOMA-IR), systolic and diastolic blood pressure and were non significantly correlated with and HOMA B% and Secret -HOMA**.**

Table 3.49: Association of IL 10 C592A polymorphism with Glycemic and Clinical Parameters in the Study Subjects

Variables	Genotype	Genotype	F -Value	p-value
FBS[a,d] (mmol/L)	CC	CA**		
		AA**		
	CA	CC**	30.95	<0.001
		AA**		
ABF[a,d] (mmol/L)	CC	CA**		
		AA**	31.38	<0.001
	CA	CC**		
		AA**		
HBA_1C[a,d] (%)	CC	CA*		
		AA**		
	CA	CC*	24.88	<0.001
		AA**		
HOMAB% [a,d]	CC	CA		
		AA		
	CA	CC	3.28	>0.05
		AA		
Secret HOMA[a,d]	CC	CA		
		AA		
	CA	CC	3.64	>0.05
		AA		
Insulin[b,c] (µU/L)	CC	CA*		
		AA*	3.02	<0.05
	CA	CC*		
		AA		
HOMA IR[b, c]	CC	CA*		
		AA**	9.31	<0.001
	CA	CC*		
		AA		
SBP[b,c] (mm Hg)	CC	CA		
		AA**	12.06	<0.001
	CA	CC*		
		AA*		
DBP[b,c] (mm Hg)	CC	CA*		
		AA		
	CA	CC*	9.97	<0.001
		AA		

Here, the a=Welch ANOVA test, since data violated homogeneity, b=One-way ANOVA Test since data confirmed homogeneity, and c=LSD test since data confirmed homogeneity, d=Dunnett Test since data violated homogeneity; *= POST HOC p<0.05; **= POST HOC p<0.001.

Table 3.50 showed the association of IL10 C592A polymorphism with lipidemic and clinical parameters in the study population. It showed that polymorphic (CC, CA, AA) variants of IL10 C592A polymorphism were a significant association with waist-hip ratio, triacylglycerol, serum

cholesterol, LDL-C, HDL-C, and visceral adiposity index and were non significantly correlated with body mass index and age.

Table 3.50: Association of IL 10 C592A Polymorphism with Lipidemic and Clinical Parameters in the Study Subjects

Variables	Genotype	Genotype	F- Value	p-value
WHR[a,d]	CC	CA**		
		AA**	10.99	<0.001
	CA	CC**		
		AA		
TG[a,d] mg/dL	CC	CA**		
		AA**	17.35	<0.001
	CA	CC**		
		AA		
Cholesterol[a,d] mg/dL	CC	CA**		
		AA**	13.96	<0.001
	CA	CC**		
		AA		
HDL-C [a,d] mg/dL	CC	CA		
		AA**	19.10	<0.001
	CA	CC		
		AA**		
LDL-C [a,d] mg/dL	CC	CA**		
		AA**	9.04	<0.001
	CA	CC**		
		AA		
AGE[b,c] years	CC	CA		
		AA*	2.82	>0.05
	CA	CC		
		AA*		
BMI[b ,c] kg/m2	CC	CA		
		AA	0.71	>0.5
	CA	CC		
		AA		
Visceral Adiposity Index[b,c]	CC	CA		
		AA*	6.67	<0.01
	CA	CC		
		AA*		

Here, the a=Welch ANOVA test, since data violated homogeneity, b=One- way ANOVA Test, since data confirmed homogeneity, and c=LSD test, since data approved homogeneity, d=Dunnett Test since data violated homogeneity; *=POST HOC p<0.01;**=POST HOC p<0.001; TC= Total cholesterol; TG=triglyceride; HDL-C=high density lipoprotein cholesterol; LDL-C=low density lipoprotein cholesterol; VAI=visceral adiposity index.

Study of Association of

TNF-α, IL-6, and IL-10 Polymorphisms

3.7 Study of Association of All Three Genes (TNF-α, IL6, and IL10 Polymorphism)

Table 3.51 showed the association of TNF-αG238A, IL-6A597G, and IL-10C592A genotypes with different anthropometric and biochemical data parameters in the study subjects and different polymorphic variety of TNF-α 238G>A showed a significant (<0.005) association with BMI and insulin. Again, IL-6 597A>G polymorphic variety showed a significant ($p<0.05$) association with SBP, DBP, HOMA B%, and Secret- HOMA. IL-10 592 C>A mutant variants were significantly($p<0.05$) correlated with Secret-HOMA and heterozygous mutant variant CA with HOMA B%.

Table 3.51: Multinomial Logistic Regression Analysis of TNF-α G/A, IL6 A/G and IL10 C/A polymorphism with Anthropometric, Clinical, Glycemic, and Lipidemic Parameters in the Study Subjects

Gene	Genotype	p-values of anthropometric and biochemical data																
		Age	BMI	WHR	FBG	ABF	INSULIN	HOMA-IR	TAG	TC	HDL-C	LDL-C	VAI	SBP	DBP	HBA1c	HOMA B	SecHOMA
TNF-alpha	GG	.319	.005	.457	.999	.669	.511	.852	.428	.542	0.894	0.517	0.272	0.604	0.947	0.817	0.966	0.108
	GA	.236	.004	.072	.621	.639	.049	.248	.08	.155	0.818	0.236	0.008	0.256	0.747	0.266	0.103	0.155
	AA	.319	.006	.547	.999	.669	.958	.988	.817	.428	0.849	1.003	0.894	0.604	0.947	0.817	0.164	0.108
IL-06	AA	.575	.930	.194	.448	.079	.348	.343	.276	.947	0.851	0.492	0.465	0.05	0.08	0.906	0.895	0.981
	AG	.087	.892	.06	.220	.360	.241	.361	.598	.812	0.086	0.863	0.597	0.355	0.924	0.535	0.027	0.037
	GG	.575	.930	.194	.448	.079	.348	.343	.276	.947	0.947	0.851	0.492	0.465	0.05	0.08	0.026	0.041
IL-10	CC	.221	.747	.892	.515	.245	.545	.802	.597	.712	0.924	0.513	0.523	0.744	0.655	0.105	0.068	.091
	CA	.593	.198	.328	.723	.492	.438	.727	.540	.502	0.066	0.308	0.670	0.157	0.688	0.902	0.002	.001
	AA	.221	.747	.892	.515	.245	.545	1.01	.597	.712	0.924	0.513	0.948	0.057	0.655	0.105	0.072	0.040

Values were presented as Mean ±SD; FBG: Fasting blood glucose; ABF: 2 hours after breakfast; HOMA B%= Beta Cell Function; HOMA-IR: Homeostasis Model of Assessment Insulin Resistance; Secretory HOMA: Secretory Homeostasis Model of Assessment; HDL= High-Density Lipoproteins and LDL= Low-Density Lipoproteins. TC= Total Cholesterol and HBA1C: glycated hemoglobin; VAI: visceral adiposity index; SBP: systolic blood pressure; DBP: Diastolic blood pressure; p-value was obtained from individual sample t-test,*$p<0.001$; level of significance was set to $p<0.05$.

Table 3.52 showed double combinations of TNF-α G238A and IL-10 C592A gene polymorphism. Most of the cases showed a significant ($p<0.005$) association between mutant variants of TNF-α G238A and IL-10C592A polymorphism. The odds of having both mutant variants of TNF-α G238A and IL-10C592A polymorphism were 65%,86%,77%, 84%, 59%, and 64% less likely to develop T2DM compared to control groups of TNF-α G238A and IL-10 C592A polymorphism, respectively.

Table 3.52: Double combinations of TNF-α G238A and IL-10C592A Gene Polymorphisms and Their Associated Risk with Type-2 DM

TNF-αG/A+IL-10C/A	Study Subjects (700)		OR (95% CI)	p-value
	Control (n=350)	T2DM (n=350)		
GG+ CC	122	50	1 (Ref.)	
GA+ CC	28	33	0.347 (0.19 - 0.63)	<0.001
GA+CA	10	31	0.13 (0.06 - 0.29)	<0.001
GA+ CC	17	31	0.22 (0.11 - 0 .44)	<0.001
GG+ CA	24	64	0.15 (0.08 - 0.27)	<0.001
GG+ AA	14	86	0.40 (0.25 - 0.65)	<0.001
AA+ CC	50	20	1.02 (0.55 - 101.94)	>0.05
AA+ CA	13	10	0.53 (0.22 - 1.36)	>0.05
AA+ AA	22	25	0.36 (0.19 - 0.71)	<0.001

Results were expressed as number (percentage); The level of significance was set to $p<0.05$; OR=Odds ratio; CI=Confidence interval.

Table 3.53 showed double combinations of TNF-α G238A and IL-6 A597G gene polymorphism and showed a non-significant association between mutant varieties of TNFα - G238A and IL-6 A597G polymorphism in most of the cases, and in one case showed a significant ($p<0.001$) association with T2DM in the double combination of TNF alpha G238A and IL-6 A597G gene polymorphism AA+AA. Odds having a double combination of TNF alpha G238A and IL-6 A597G gene mutant (AA+AA) genotypes were 2.16 times more likely to develop T2DM compared to their homozygous normal control group, and 95% CI was (1.25 - 3.74).

Table 3.53: Double combinations of TNF-αG/A, IL-6A/G Gene Polymorphisms and Their Associated Risk with Type-2 DM

TNF-αG/A+IL-6A/G	Study Subjects (n=700)		OR (95% CI)	p-value
	Control(n=350)	T2DM(n=350)		
GG+ AA	130	108	1 (Ref.)	
GA+ AA	42	41	0.85 (0.52 - 1.39)	>0.05
GA+ AG	02	3	0.55 (0.09 - 2.75)	>0.05
GA+ GG	11	51	0.17 (0.08 - .36)	<0.001
GG+ AG	10	12	0.69 (0.28 - 1.59)	>0.05
GG+ GG	70	80	0.72 (0.47 - 1.09)	>0.05
AA+ AA	60	23	2.16 (1.25 - 3.74)	<0.05
AA+ AG	02	05	0.33 (0.06 - 1.59)	>0.05
AA+ GG	23	26	0.77 (0.39 - 1.33)	>0.05

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval.

Table 3.54 showed double combinations of IL-10 C592A and IL-6 A597G gene polymorphism and showed a significant (p<0.05) association between heterozygous and homozygous mutant varieties of TNF-α G238A and IL-10 C592A polymorphism in most of the cases. Odds having both mutant variants of IL-10C592A and IL-6A597G polymorphism were 66%, 94%,82%, 63 %, and 66% less likely to develop T2DM than control groups of IL-10C592A and IL6A597G polymorphism, respectively.

Table 3.54: Double Combinations of IL-6 A/G and IL-10C/A Gene Polymorphisms and Their Associated Risk with Type-2 DM

IL-10C/A+IL-6A/G	Study Subjects (n=700)		OR (95% CI)	p-value
	Control(n=350)	T2DM(n=350)		
CC+ AA	132	44	1 (Ref.)	
CA+ AA	33	33	0.33 (0.18 - 0.60)	<0.01
CA+ AG	1	5	0.06 (0.05 - 0.51)	<0.01
CA+ GG	13	37	0.17 (0.05 - 0.24)	<0.001
CC+ AG	10	6	0.55 (0.20 - 1.64)	>0.05
CC+ GG	58	53	0.36 (0.21 - 0.59)	<0.001
AA+ AA	67	66	0.33 (0.21 - 0.54)	<0.001

Results were expressed as number (percentage); The level of significance was set to p<0.05; OR=Odds ratio; CI=Confidence interval.

Table 3.55 showed triple combinations of three (TNFα G238A+IL6A597G+IL10 C592A) gene polymorphisms and their associations with T2DM, and it showed their homozygous and heterozygous mutant variants were significantly ($p<0.05$) associated with T2DM. The odds of triple combinations of 3 gene polymorphisms were 55%, 94%, 89%, 87%,77%, 88%, 95%, 97%, and 82% less likely to develop T2DM compared to their homozygous normal gene, respectively.

Table 3.55: Triple Combinations of TNF-αG/A, IL-6 A/G, and IL-10 C/Agene polymorphisms and their associated risk with T2DM

TNFα G/A+IL6A/G+IL10 C/A	Study Subjects (n=700)		OR (95% CI)	p-value
	Control(n=350)	T2DM (n=350)		
GG+GG+CC	37	25	0.45 (0.23- 0.88)	<0.05
GG+GA+CA	1	5	0.06 (0.05 -1.51)	<0.05
GG+AG+AA	2	6	0.10 (0.02- 0.45)	<0.01
GG+GG+AC	10	24	0.12 (0.05- 0.31)	<0.001
GG+GG+AA	23	31	0.22 (0.11 -0.45)	<0.001
GG+AA+AC	13	35	0.11 (0.05 -0.25)	<0.001
GG+AA+AA	39	49	0.24 (0.12 -0.45)	<0.001
GA+GG+CC	7	18	0.11 (0.05- 0.37)	<0.001
GA+GG+CA	2	11	0.05 (0.01 -0.27)	<0.001
GA+GG+AA	2	22	0.02 (0.06 -0.11)	<0.001
GA+AA+CA	8	20	0.12 (0.04 -0.31)	<0.001
AA+GG+AA	8	14	0.17 (0.06 -0.45)	<0.01

Results were expressed as number (percentage); The level of significance was set to $p<0.05$; OR=Odds ratio; CI=Confidence interval.

Table 3.56 showed the haplotype of single nucleotide polymorphisms of TNF-α G238A, IL-6A597G, and IL-10 C592A for association with type 2 DM, and it showed GAA haplotype was significantly ($p<0.05$) associated with the development of type 2DM. Odds with GAA haplotype was 2.01 times more likely to develop T2DM than control, and 95% CI was (1.58-2.57). GAC haplotype showed a significant ($p<0.05$)association with T2DM, and odds with GAC haplotype was 53% less likely to develop T2DM than control.

Table 3.56: Haplotype of SNPs of TNFα-G238A, IL-6A597G, and IL-10C592A for Association with Type 2 DM

Haplo type	Study Subjects (n=700)		Chi2	OR (95% CI)	Pearson's p-value
	T2DM (n=350) n (%)	Control (n=350) n (%)			
GAA	232 (0.33)	138 (0.19)	32.45	2.01 (1.58-2.57)	<0.05
AAC	127 (0.18)	150 (0.21)	2.38	0.81 (0.62- 1.05)	>0.05
GGA	74 (0.10)	55 (0.07)	3.08	1.38 (0.96-1.99)	>0.05
GGC	66 (0.09)	85 (0.12)	2.67	0.75 (0.53-1.05)	>0.05
GAC	118 (0.16)	212 (0.30)	35.03	0.46 (0.36-0.60)	<0.05
AAA	83 (0.11)	60 (0.08)	4.12	1.43 (1.01-2.03)	>0.05

Results were expressed as number (percentage); The level of significance was set to $p<0.05$; OR=Odds ratio; CI=Confidence interval.

4.1 Discussion

Diabetes has become one of the common non-communicable diseases with a high prevalence and incidence. A meta-analysis in our country demonstrated that the occurrence of diabetes in adults is uprising steadily, from 4% in 1995-2000 to 5% in 2001-2005 then to 9% in 2006-2010, respectively (Akter et al., 2014; Khan et al., 1998).

Numerous researchers carried out various studies during the last few years to identify genes that are responsible for T2DM (O'Beirne et al., 2018). Using candidate gene analysis and GWAS, investigators have identified several potential susceptible genes of T2DM. This research leads to retrospective research to estimate the correlation of TNF- α, IL-6, and IL-10 gene polymorphisms with T2DM in the Bangladeshi population. To the extent of my perception, my research was the opening effort to evaluate the polymorphisms of TNF- α, IL6, and IL10 genes with T2DM in the Bangladeshi population.

One of the significant risk factors for T2DM is obesity. Medical findings demonstrate a significant correlation of T2DM with abdominal fatness than peripheral obesity (Kamath et al., 2011). Roth et al. (2017) discovered a strong positive association between fatness and T2DM. However, the current research showed a non-significant ($p>0.05$) mean difference with BMI and WHR and significant ($p<0.05$) mean differences in blood pressure (systolic and diastolic) between the T2DM and control subjects (Table 3.1).

This study showed that the graduate and postgraduate education level was significantly higher ($p-<0.05$) in control than in type 2 diabetes mellitus. We also found that fasting insulin value, insulin resistance, and HOMA B% were significantly elevated in T2DM than in control, shown in table 3.2. A study of India's heart watch demonstrated that diabetes awareness, treatment, and control are lower among the low educational status. Zhang et al. (2015) concluded that the low academic level has a greater prevalence of high carbohydrate intake and had more insufficient diabetes control. This study supported Zhang's investigation (Table 3.2). Werth et al. (2000) and his associates Zuo et al. (2014) provided evidence that the incidence of DM was found to be higher in urban than rural areas. Similar to their findings, this research also noticed that diabetic subjects were significantly higher in urban settings, whereas the control participants were more in the rural population (Table 3.2).

Xie et al. (2009) discovered a significant connection between cigarette smoking and diabetes. Upon analysing the outcome of the conducted research, the control group's smoking status was not significantly ($p>0.05$) lower than the T2DM subjects. This observation is contradictory to the results of Xie et al. (2009). Silva et al. (2014) suggested that the existence of diabetes in the family is a crucial risk element for the incidence of T2DM; however, the causes influencing these excess risk factors are ill-defined. Similar to their findings, this study also found a strong association ($p<0.05$) between the two factors, as expected (Table 3.3).

Bergman et al. (2002) suggested that the primary determinant of T2DM is the inactivity of the pancreatic β-cell to reimburse for insulin resistance. However, other researchers Unnikrishnan et al. (2014), Abdul-Ghani et al. (2010), and Weyer et al. (1999) also found an association of distinguishing metabolic syndrome of T2DM with higher insulin level, a significant rise of insulin resistance, as well as an increasing occurrence of Diabetes. In contrast to their findings, the present research showed that FBG, ABF, HbA1c%, HOMA B%, secretory HOMA, and HOMA IR levels were significantly ($p<0.001$) increased in T2DM subjects (Table 3.4). However, Kashyap et al. (2003) concluded that insulin inactivity developed due to hyperlipidemia showed a decisive role in the occurrence of T2DM. Moreover, Wong et al. (2013) reported that T2DM was separately related to increased plasma TAG, decreased good cholesterol HDL, and an elevated level of bad cholesterol (LDL-C). In this research, the TAG and VAI were remarkably ($p<0.00$) higher in T2DM in comparison to the monitoring group, which is the same as the study of Petrie, 1984 and Wong et al. (2013) (Table 3.4).

In humans, the TNF-α gene is related to increased BMI, the proportion of body fat, and high blood insulin level, whereas weight reduction caused decreased TNF-α level (Pokharel et al., 2017; Sarkar and Meshram, 2017; VinodMahato et al., 2011). The high appearance of TNF-α in fat tissue had been observed to increase insulin inactivity. The gene polymorphism at position -238 G>A in the promoter region of TNF-α was noticed to augment the gene's translation in fat tissues (Cawthorn and Sethi, 2008; Rabinovitch, 1998).

Furthermore, in 2014, an aggregated analysis was carried out by Zhao et al. (2014), which implicated that the TNF-α -308A genotype could be a risk issue for the occurrence of T2DM in an Asian population. At the same time, Golshani et al. (2015) reported that TNF-α -308 GA and

AA genotypes were correlated with a greater possibility of type-2 diabetes development in the Iranian subjects.

Guzmán-Flores et al. (2011) mentioned that mutant AA genotype of TNF-α (G238A) polymorphism was significantly correlated with T2DM in Mexican. In contrast, they did not find any significant correlation with TNF-α (G308A) polymorphism. In this study, Table 3.7, the Homozygous mutant variant (AA) of the TNF-α (G238A) genotype showed significant ($p<0.0001$) association with T2DM, and mutant "A" allele frequency was significantly linked to the possibility of development of T2DM. The results of this study were similar with Guzmán-Flores et al. (2011). In another Pima Indians study, Fernandez-Real et al. (1997) concluded that TNF-α (G238A) gene polymorphism is correlated with overweightness and insulin resistance in T2DM subjects when it was considered in the recessive model, which was similar to our findings (Table 3.8). This observation is contradictory to the findings of Walston et al. (1999), Lee et al.(2000), Romeo et al.(2001), and Koch et al. (2000). They reported that mutant allele and genotypes GA and AA were significantly associated with T2DM in the dominant model.

Moreover, in this research, homozygous (AA) mutant variants of TNF- α showed a significant ($p<0.001$) association with type 2 male diabetics (Table 3.9). Swaroop et al. (2012) and Jellema et al.(2004) mentioned that TNF-α (G238A) polymorphism might act as a potentially critical pathophysiological part in the progress of insulin inactivity, particularly in males and in people with high BMI.

The study showed that the polymorphic variants of Tumor Necrosis Factor-alpha (G238A) polymorphism were significantly associated with FBG, ABF (Table 3.19). The outcome of the study was in agreement with the findings of Valenti et al. (2002), Hawrami et al. (1996), and Shiau et al. (2003).

Besides, this study explored the relation of lipidemic and clinical parameters with TNF –alpha (G238A) polymorphism in T2DM. Moreover, in this research, the polymorphic variants of TNF alpha were significantly correlated with waist-hip ratio, serum cholesterol, LDL-C, HDL-C, diastolic blood pressure, and non-significantly correlated with age, body mass index, triacylglycerol, systolic blood pressure, visceral adiposity index (Table 3.20). Earlier, Saxena et

al. (2013) also reported an association of TNF alpha (G238A) gene polymorphism with type 2 diabetes and dyslipidemia in the North Indian population and concluded that Mutant “A” allele increases the expression of TNF-alpha in fat tissue.

Guo et al. (2012) and Pan et al. (2011) have found that insulin resistance, along with metabolic changes such as hyperglycemia and dyslipidemia, can gradually increase in those Japanese subjects, on the presence of the IL6 (A597A) polymorphism. Furthermore, the association of the IL6 gene with other pro-and anti-inflammatory cytokine genes is involved in pancreatic beta-cell destruction, which may give rise to insulin resistance.

Saxena et al. (2009) reported that polymorphism in the promoter region (-597) of the IL-6 gene is associated with Diabetes and metabolic disorder in the Asian population. Table 3.22 indicated that the homozygous mutant variant (GG) of the IL-6 (A597G) genotype has a strong association with T2DM ($p<0.001$). Moreover, the frequency of the mutant “G” allele was constituently ($p< 0.001$) 3.75 times elevated in diabetic subjects, in contrast, to control when the frequency of the “A” allele was considered as the reference allele. This study also found both mutant variants (GG, AA) of IL-6 (A597G) were found to be significantly linked to T2DM in both sexes (Table 3.24 and Table 3.25). The findings of this research were similar to those of Saxena et al. (2009) and Nishimura et al. (2009). They concluded normal “A” allele might be a protective factor against diabetes mellitus.

Kubaszek, (2003) suggested that the Single Nucleotide Polymorphism of IL-6 (G176C) was not related to T2DM in the Finnish population. However, another researcher, Fernández-Real et al. (2000), analyses a small group of the population of native Americans and Spanish Caucasians, which showed that the 'G' allele of IL-6 (G174C) polymorphism could be correlated with a greater risk of T2DM.

Maitra et al. (2008) mentioned that IL-6 (A597G) polymorphism was significantly associated with CAD and diabetes in the Chinese people. This research investigated the association of IL-6 (A597G) polymorphism with glycemic parameters in the study subjects and showed polymorphic variants of IL-6 (A597G) polymorphism were significantly correlated with fasting blood glucose, 2 hours after breakfast, glycated hemoglobin (HBA_1C), insulin, the homeostatic

model of insulin resistance (HOMA-IR) (table 3.34). Moreover, Table 3.35 presented the polymorphic variants of IL-6 (A597G) polymorphism were significantly correlated with serum cholesterol, HDL-C, visceral adiposity index, waist-hip ratio, and systolic blood pressure and were non-significantly correlated with age, BMI, triacylglycerol, diastolic blood pressure (DBP), and low-density lipoprotein (LDL-C). The outcome of this research was consistent with the results obtained from the Chinese population.

Table 3.37 showed the IL-10 (C592A) genotype frequencies and estimated risk of T2DM. Here, both the homozygous mutant variant (AA) and heterozygous mutant (CA) of the IL-10 genotype presented an important correlation with T2DM ($p<0.001$). The distribution of the "A" allele was significantly ($p<0.001$) higher in diabetic subjects, in contrast to control. The outcome of this research was in line with the Japanese population (Ide et al., 2003). They concluded that IL-10 (C592A) polymorphism reduces insulin sensitivity and causes diabetes and metabolic syndrome in the Japanese people.

It was also found that both the dominant and recessive model analysis of IL-10 (C592A) polymorphism, the frequency of CA+AA genotype was significantly ($p<0.001$ correlated with T2DM, and the odds were 4.058 many times the likelihood of developing T2DM in the cases of having CA or AA genotype compared to CC genotype (Table 3.38). Our findings were in line with those of the North Indian population (Singhal et al., 2015). They concluded that if the "A" allele is dominant, then the expression of the IL-10 gene increased in lean fat tissue.

Table 3.40 showed the polymorphic variants of IL-10 C592A polymorphism were significantly associated with waist-hip ratio, triacylglycerol, serum cholesterol, LDL-C, HDL-C, visceral adiposity index and were not significantly correlated with body mass index and age. Pereira's study followed up these results (Pereira et al., 2015). Furthermore, T2DM was associated with both homozygous (AA) and heterozygous (CA) mutant variants of the IL-10 C592A polymorphisms in both sexes in this research (Table 3.39 and Table 3.40). These findings were not consistent with others (Zheng et al., 2014; Wensveen et al., 2015).

Table 3.41 showed that all age ranges of female participants with IL-10 C592A polymorphism were significantly associated with T2DM. Furthermore, various studies refer that serum levels of the anti-inflammatory marker IL-10 were higher in grade-3 obese females without metabolic

complications and that decreased IL-10 levels were correlated with the Metabolic Syndrome (Esposito et al., 2003; Wilkinson et al., 2014). In various studies, the role of IL-10 gene polymorphisms in metabolic complications was demonstrated by several authors amidst a variety of populations, for example, T2DM, overweightness, and insulin resistance (Mohebbatikaljahi et al., 2009). Moreover, they predict that these genetic variants also add to the expression of a specific gene, and posttranscriptional control could also have an impact on its ultimate protein synthesis (Scarpelli et al., 2006; Winer et al., 2009).

Table 3.49 offered polymorphic variants of IL-10 gene polymorphism was an important association with fasting blood glucose, 2 hours after breakfast, glycated hemoglobin (HBA1C), fasting insulin, the homeostatic model of insulin resistance (HOMA-IR), systolic and diastolic blood pressure and were non significantly associated with HOMA B% and Secret-HOMA. Some other research demonstrated the absence of correlation of serum IL-10 level with IL-10 (C592A) polymorphisms (Roh et al., 2002; Xu et al., 2013). However, due to the presence of other IL-10 lowering factors in the system, there was no correlation between serum levels of IL-10 and its gene polymorphisms, favoring inflammation caused metabolic syndrome (Ji et al., 2003; Lin et al., 2005).

Furthermore, Table 3.49 showed polymorphic variants of IL-10 polymorphism was a significant association with fasting blood glucose, 2 hours after breakfast, glycated hemoglobin (HBA1C), fasting insulin, the homeostatic model of insulin resistance (HOMA-IR), systolic and diastolic blood pressure, and were non significantly correlated with HOMA B% and Secret -HOMA. The results of this research were similar to those of a North Indian study (Vanexel et al., 2003; Tong et al., 2017).

Along with this, another reviewing research was directed by Zhuanping et al. (2016), in which no potential risk for T2DM progress correlated with TNF-1082 GA+GG and IL 10-592 AC+AA genotypes was found. The outcome of Zhuanping et al.'s study was due to the small sample size and various genetic backgrounds. Larger-scale genome studies are required to evaluate these relations further.

Our research findings revealed that genotypes and alleles were significantly related to T2DM manifestation in double and triple combinations in the study population (Table 3.53, Table 3.54,

and Table 3.55). The outcome of this research was correlated with those of Saxena et al. (2009) but not with those of Knights et al. (2014).

Yin et al. (2012) and Singhal et al. (2015) reported that GAA haplotype is high in Asian populations with T2DM and metabolic disorders, whereas it is low in the Caucasian population. In our haplotype (Table 3.56) analysis, we observed that the GAA haplotype significantly ($p<0.05$) was associated with the development of type 2DM in the Bangladeshi population. Our findings were similar to those of Yin et al. (2012) and Singhal et al. (2015).

Therefore, there are some possible reasons for the variance between the previous studies and our study. This may be due to ethnic and racial differences, including diverse social or cultural factors which might have influenced the results.

The outcome of our conducted study showed that only homozygous mutant variant of TNF-α, as well as IL-6 gene polymorphisms, were significantly ($p<0.001$) associated with T2DM, whereas IL-10 gene polymorphism was significantly ($p<0.001$) correlated with T2DM in both homozygous and heterozygous mutant variant. The study outcomes recommend that specific GAA haplotype was significantly ($p<0.05$) correlated with T2DM.

From the obtained results, it can be concluded that TNF-α, IL-6, and IL-10 gene polymorphisms are strongly associated with T2DM in the population of Bangladesh.

4.2 Limitations of this study:

There are some limitations to the study. The following limitations of the study may be considered for interpretation of the results:

1. Complete genome sequencing will be important for methodical documentation of potentially responsive mutations in the TNF-α, IL-6, and IL-10 whole-genome functional regions.

2. However, another limitation of the current research is the relatively limited sample size utilized for TNF-α, IL6, and IL10 gene polymorphisms analysis, which may render the false correlations by any means or may fail to detect actual differences.

3. The study was a hospital-based case-control study; the T2DM cases and controls were recruited from the hospital, so a selection bias may occur.

4. Since the study participants were recruited from Dhaka, Bangladesh, so they are not representative of the Bangladeshi population as a whole.

Ethnic differences may contribute to finding different results in different studies since both genetic and environmental factors (e.g., diet, lifestyle) are important in the development of T2DM, as well as their complex interactions. It is still unknown how environmental factors like lifestyle and obesity may contribute to the association of genotype and T2DM. However, it is important to note that the current research is the first to investigate the association between the aforementioned TNF- α, IL-6, and IL-10 gene SNPs and T2DM in the Bangladeshi population.

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Appendix:

Consent Form (In English)

I am DR. RoksanaYeasmin, want to take your consent regarding your disease for research purpose.

Purpose of the Research: Diabetes and its vascular complications are increasing day by day. HbA1c is essential for the diagnosis and prognosis of Diabetes. The molecular basis of DM is also important for the future makeup and management of DM. If this research is successful, then we will know whether polymorphism is responsible for DM or not. For this purpose, I want to take 5 ml of blood, and some of your personal information, which will be concealed, and this information will be used for research purposes. If you want, then you can join, or if you don't want, then back out from the study. For this research purpose, you need not pay.

Signature of the Participant **Signature of the Researcher**

সম্মতিপত্র

গবেষনারবিষয়

TNF-alpha, IL-6 and IL-10 জীনেরপলিমরফিজমেরকারনেবাংলাদেশে টইপ ২ ডায়াবেটিসহওয়ারপ্রবনতানির্ণয়।

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, কিছুকিছুঔষধেরকার্যকারিতাসম্পর্কিতজীনেরভিন্নতাবাপলিমরফিজমেরকারনেমূখেসেবনীয় ঔষধগুলোসঠিকভাবে ডায়াবেটিক নিয়ন্ত্রণে ভূমিকারাখতেপারেনা। এই গবেষণায় দেখা হবে যে, ডায়াবেটিক রোগীদের তিনটিজীনTNF-alpha, IL-6 GবংIL-10এরপলিমরফিজমডায়াবেটিসহওয়ারঝুকিবাড়ায়কিনাএবং ডায়াবেটিস নিয়ন্ত্রণে এইসবজীনেরআদৌকোনসম্পর্কআছেকিনা।

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, কোলেস্টেরল, ট্রাইগিসারাইড, এইচ ডি এল, এল ডি এল, ক্রিয়েটনিন, 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

অংশগ্রহণকারীর স্বাক্ষর

A pretest was done before finalizing the structured questionnaire of this study.

QUESTIONNAIRE AND DATA COLLECTION SHEET

TITLE: Association of TNF-alpha, IL-6 and IL-10 gene polymorphisms with type 2 DM in relation to insulin secretion and action in Bangladeshi population

Date:

Name of the respondent……

ID……

…

Address

……

……

Contact no

SociodemographicVariables:

……

…….

Age……years

Sex……..(Male=1, Female=2)

Monthly income…….Taka

Occupation sedentary worker/Active worker

Dietary history: fast food taking history/ week : 1)Yes 2) No 3) Occasional

Exercise history: 1) Yes if yes a) 30 minutes b) 45 minutes c) 60 minutes 2) No

Educational history: 1) Primary 2) Secondary 3) Higher secondary 4) graduate 5) Postgraduate

Smoking history: a) Yes b) No

History of

Anemia: yes/no,

Duration……years.

Diabetes: Yes/ No, if yes, duration……years.

Family history of DM: Yes/no, if yes, then father, mother, uncle, aunt, grandfather, or mother.

Hypertension: Yes/ No, if yes, duration……years.

Renal disease: Yes/ No, if yes, duration……years.

Liver disease: Yes/ No, if yes, duration……years.

Drug history: Yes/ No, if yes, duration……years.

Anthropometry and Examination

Height (in meter)…….

Weight(in kg)…….

BMI……

Waist circumference……

Hip circumference……

Waist: hip (ratio)…….

Systolic BP (mm of hg)…….

Diastolic BP (mm of hg)…….

Pulse (rate/min)……

Anemia…….

Jaundice: ……

Biochemical parameters:

1. Fasting Blood glucose…….mmol/l
2. HbA1c(HPLC)……mg%
3. Lipid profile
4. Total cholesterol……..mg/dl
5. Triglyceride…….mg/dl
6. LDL (cholesterol)……mg/dl
7. HDL(cholesterol)……..mg/dl.
8. IL-10 gene polymorphism……..
9. TNF gene polymorphism……..
10. 2ABF……mmol/l
11. Serum insulin level……mmol/l
12. HOMA-IR
13. HOMA B%
14. Secret HOMA
15. VAI

Comment……
……

লিখিতপ্রশ্নাবলী

1| রোগীরনাম t

.....

2| AvBwWbs t

.....

3| ডায়াবেটিক রেজিঃনং t

.....

4| wVKvbv t

.....

5| eqm t

.....

6| wj½ t K) ciæl L) gwnjv

7| বাসস্থান t K) mf¨/wkó L) kni M) Mvg

8| শিড়্গাগত যোগ্যতা ঃ

K) Awkw ÿ Z L) cv_wgK M) gva¨wgK ঘ) উচ্চ মাধ্যমিক 0) গ্রাজুয়েশনবাঅধিক

9| পেশা ঃ K) KgRxex L) e¨emv গ) গৃহকর্মী

10| কাজের/পেশারধরন t

.....

11| KwqKcwikg t nÖ/bv

K) Kg wegL (30 wgwbU) L) Aícwikg (30-60 wgwbU) M) ga¨gcwikgx (60-120 wgwbU)

ঘ) বেশীপরিশ্রমী (১২০ মিনিট +)

12| gwmKAvq t

.....

পরিবারেরসদস্য সংখ্যা ঃ .....

13| ডায়াবেটিসনির্ণয়ের সময়বয়স t .....

14| পারিবারিকডায়াবেটিসেরবর্ণনা t

.....

15| agcvqxwKbv ? K) nÖv L) bv

16| g`¨cvqxwKbv? K) nüvi L) bv

17| ঔষধেরহিসাব t

.....

18| এ্যানথ্রোপুমেট্রিকপরিমাপt

K) IRb ..... খ) উচ্চতা ..... গ) বিএমআই .....

19| i³Pvc t .....

20| বায়োকেমিক্যালপ্যারামিটার

K) GBPweG Iqvbwm .....

খ) খালিপেটে রক্তের সুগার .....

গ) ভরাপেটে রক্তের সুগার .....

ঘ) লিপিড প্রোফাইল

- টোটাল কোলেস্টেরল .....
- UvBwMømvivBW .....
- এলডিএল কোলেস্টেরল .....

- এইচ ডিএল কোলেস্টেরল .....

ঙ) সিরাম ক্রিটেনিন .....

P) GBP‖e% .....

পরিদর্শকের স্বাক্ষর ও তারিখ

বাংলাদেশ ডায়াবেটিক সমিতি
DIABETIC ASSOCIATION OF BANGLADESH

Memo No. BADAS-ERC/EC/15/00231 Date: July 6, 2015

Subject: Ethical Clearance

The Ethical Review Committee (ERC) of the Diabetic Association of Bangladesh (BADAS) has approved the under mentioned protocol:

Title of the Research Work	:	Association of TNF- α, IL-6 and IL-10 Gene polymorphism with Type 2 Diabetes Mellitus in Relation to Insulin Action and Secretion
Principal Investigator	:	Dr. Roksana Yeasmin Associate Professor Department of Biochemistry and Molecular Biology Ibrahim Medical College Shahbag, Dhaka-1000
Guide	:	Prof Yearul Kabir PhD Department of Biochemistry and Molecular Biology, University of Dhaka
Co-Guide	:	Prof Dr. M.A Muttalib Professor of Biochemistry Chief Scientific Officer
Place of Study	:	BIRDEM Hospital
Study period	:	Two years from the date of issuance of this letter

K. M. S. Aziz

(Dr. KMS Aziz)
Chairman
Ethical Review Committee
Diabetic Association of Bangladesh

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