Investigation of anti-glycation role of Glyoxalase 1 (GLO1) gene with increase susceptibility to Type II Diabetes Mellitus

Faria Shahid

00000362527

Prof. Dr. Attya Bhatti

ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES (ASAB)

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY (NUST)

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Faria Shahid

Registration no. 00000362527

MS Healthcare Biotechnology

Supervisor

Prof. Dr. Attya Bhatti

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NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY (NUST)

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We hereby recommend that the dissertation prepared under our supervision by Faria Shahid (362527) titled "Investigation of anti-glycation role of Glyoxalase 1 (GLO-1) gene with increased susceptibility to Type II Diabetes.", be accepted in partial fulfillment of the requirements for the award of MS degree in Healthcare Biotechnology with A grade.

Examination Committee Members

1. Prof. Dr. Peter John — Signature:

2. Asst. Prof. Dr. Miriam Kathleen Gomez — Signature:

4. Prof. Dr Iram Murtaza — Signature:

Supervisor's name: Prof. Dr. Attya Bhatti — Signature:

Prof. Dr. Attya Bhatti
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Date: 25th Aug, 2023

Dr. Sobia Manzoor
Tenured Professor
Head of Department (HoD)
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Date: 30-08-23 — Head of Department

COUNTERSINGED

Prof. Dr. Muhammad Asghar
Principal
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Date: 01-09-23 — Dean/Principal

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Certified that final copy of MS thesis entitled "**Investigation of anti-glycation role of Glyoxalase 1 (GLO1) gene with increase susceptibility to Type II Diabetes Mellitus"** submitted by Faria Shahid (Registration # 00000362527), of Atta-ur-Rahman School of Applied Biosciences (ASAB), has been verified by undersigned, found that complete in all respects as per NUST regulations, and is accepted as fulfilment for the award of MS degree.

Prof. Dr. Attya Bhatti
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied
Biosciences (ASAB), NUST Islamabad

Supervisor: _____

Prof. Dr. Attya Bhatti

Atta-ur-Rahman School of Applied Biosciences,

National University of Sciences and Technology.

Dr. Sobia Manzoor
Tenured Professor
Head of Department (HOD)
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied
Biosciences (ASAB), NUST Islamabad

Head of Department: _____

Prof. Dr. Sobia Manzoor

Atta-ur-Rahman School of Applied Biosciences,

National University of Sciences and Technology.

Prof. Dr. Muhammad Asghar
Principal
Atta-ur-Rahman School of Applied
Biosciences (ASAB), NUST Islamabad

Principal: _____

Prof. Dr. Muhammad Asghar

Atta-ur-Rahman School of Applied Biosciences,

National University of Sciences and Technology.

Dated: 01-09-23

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Prof. Dr. Attya Bhatti
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied
Biosciences (ASAB), NUST, Islamabad

Supervisor: _____

Prof. Dr. Attya Bhatti

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Faria Shahid

Faria Shahid

00000362527

DEDICATION

All my effort is dedicated to “My beloved family”.

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I am grateful to Allah Almighty, my supervisors, lab colleagues, seniors, friends and ASAB for their support during this research. I am thankful to my parents for making me able to stand where I am now.

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LIST OF ACRONYMS

AGEs	Advanced glycation end products
ADA	American Diabetes association
FPG	Fasting plasma glucose
GLO1	Glyoxalase 1 gene
Glo1	Glyoxalase 1 enzyme
HLA	Human leukocyte antigen
HRM	High resolution melting analysis
IDF	International Diabetes federation
IRB	Institutional review board
MGO	Methylglyoxal
OGTT	Oral glucose tolerance test
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism
T2DM	Type II Diabetes Mellitus
TFBS	Transcription factor binding site
WHO	World health organization

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ABSTRACT

Type II Diabetes Mellitus (T2DM) is a global cause of increase mortality and morbidity. It is characterized mainly by high glucose index (hyperglycaemia). Moreover, hyperglycaemia is involved in enhanced accumulation of advance glycation end products (AGEs) in body. Detoxification of advanced glycation end products is carried out by glyoxalase system. Glyoxalase system maintains homeostasis of advance glycation end products (AGEs) by recycling methylglyoxal (MGO; a precursor to AGEs formation). The conversion of MGO to D-lactate is carried out chiefly by two enzymes. Glyoxalase 1 (Glo1) is given more importance owing to its rate limiting ability. Genetic variations like single nucleotide polymorphisms (SNPs) in the enzyme coding or regulatory genes like in Glyocalase1 gene (GLO1) might disturb the functional aspect of enzyme Glo1. This research study aimed to use computational bioinformatic tools and High-Resolution Melting (HRM) analysis to predict and validate functionally significant SNPs in GLO1 gene associated with T2DM. For computational analysis of non-coding regulatory single nucleotide polymorphisms (SNPs), data was collected and analysed from online databases and web-based tools. *In-silico* screening gave seven SNPs expected to affect gene function and advance glycation end products accumulation (AGEs), increasing susceptibility to Type II Diabetes Mellitus. Genetic association studies for glyoxalase 1 gene SNPs were done by High-Resolution Melting analysis. High Resolution melt curve analysis of Glyoxalase 1 gene SNP rs1038747749 authenticate its significant association with disease pathogenesis, whereas SNP rs753587598 showed no significant association, further studies can be done to check their association with large sample size.

INTRODUCTION: TYPE II DIABETES MELLITUS

Type II Diabetes Mellitus (T2DM), also known as non-insulin dependent diabetes is becoming a global cause of increase mortality and morbidity (World Health Organization, 2018). Increase rate of mortality is associated with early appearance and late diagnosis, especially in underdeveloped countries. More than 80% (~90%) of patients suffering from diabetes are type II diabetic. T2DM is marked by constant glucose level elevation (hyperglycaemia), insulin resistance and deficiency and pancreatic cell functioning. Environment, genetics and behaviour are various factors contributing to the early advent of T2DM (Olokoba et al., 2012). Two primary conditions leading to T2DM are deprivation of insulin released by β-cells and insufficient uptake by resistance tissues (having decrease sensitivity to insulin) (Galicia-Garcia *et al*., 2020). Constant hyperglycaemic condition in T2DM is hypothesized for causing macrovascular complications.

1.1 Prevalence of Type II Diabetes Mellitus

Type II Diabetes Mellitus accounts for 90% of the cases suffering from diabetes. World Health Organization (WHO) reported 3% rise in mortality rate across globe from 2000 – 2019. Moreover, in under developing countries mortality rate is 13%. According to prevalence studies men are more prone to T2DM than women (22.2% and 14.0% respectively) (Magalhães *et al*., 2023).

Prevalence calculation for First Nation individuals suggests more incidence increase of T2DM in younger generation (age < 30) as compared to older people (age > 30)(González *et al*., 2009; Ruth *et al*., 2023). Overall, prevalence of T2DM is increasing worldwide, steady increase in incidence rate of T2DM in older age group is observed in population of Saudi Arabia (Jarrar *et al*., 2023). According to International Diabetes Federation (IDF), prevalence of diabetes will be increased 12.8% worldwide by 2024.

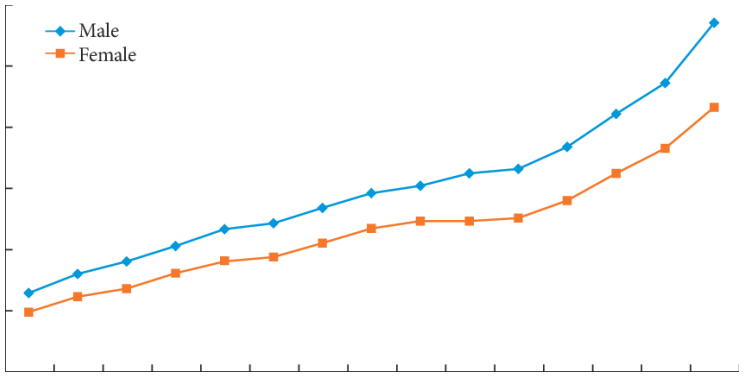

Figure 1: Gender based prevalence of T2DM in Korean population (Hong et al., 2021).

1.2 Pathophysiology

Equilibrium between glucose production from pancreatic β-cells and consumption by body cells is necessary for the maintenance of glucose levels in body. Disruption in these mechanisms can lead to development of Type II Diabetes Mellitus. Three main sources of glucose in blood are: digestion of carbohydrates, gluconeogenesis and glycogenolysis. Consumption of carbohydrates can lead to lead to increase in glucose level, consecutively suppressing gluconeogenesis. However, fasting can lead to equal level of gluconeogenesis and glycolysis (Henquin *et al*., 2017). The main factors linked with pathophysiology are insulin resistance and malfunctioning of pancreatic cells (shown in Figure 2) (Javeed & Matveyenko, 2018).

1.2.1 Peptide hormone secretion from Pancreas

Pancreatic α-cells and β-cells are involved in the production of peptide hormones glucagon and insulin respectively. Insulin and glucagon are mainly responsible for maintaining blood glycaemic levels. Insulin promotes absorption of glucose, simultaneously suppressing its neogenesis. Under normal conditions, 30-70 units of insulin is released from β -cells per day and it is mainly controlled by glucose levels. Glycogen reduces absorption and increase glycolysis, releasing more glucose into blood stream. Abnormal functioning of pancreatic β-

cells can cause increase in glucose concentration leading to diabetes (Marchetti *et al.*, 2020; Prato & Marchetti, 2004).

1.2.2 Insulin Resistance

Insulin resistance is characterized by inability of target tissues to utilize glucose when stimulated by insulin. Insulin is mainly responsible for the internalization of blood glucose by interacting with cell receptors (mainly on liver, skeletal muscle and adipose tissue). Lack of glucose signals for more insulin production and release into blood stream. Constant elevation of insulin can result in metabolic complications including obesity, cardiovascular diseases and Type II Diabetes Mellitus (Paneni *et al.*, 2014; Wondmkun, 2020).

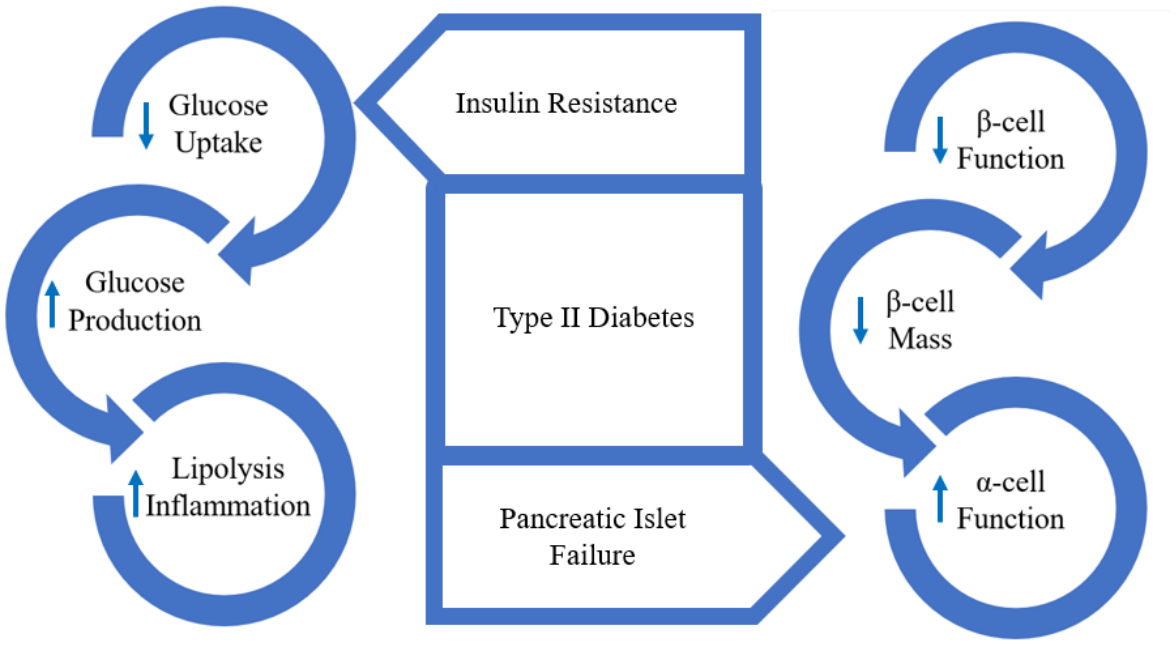

Figure 2: Pathophysiology of Type II Diabetes Mellitus.

1.3 Risk factors:

Diabetes of Type 2 is a heterogeneous disease, its incidence is triggered by variables including genetic and environmental factors (Dendup *et al.*, 2018).

1.3.1 Genetic factors

The pathophysiology of insulin resistance has not been clear yet, but it is investigated that genetic factors play role in onset of T2DM. T2DM, a polygenic disorder proved by segregation analysis, is termed to have several susceptible loci located by genome wide association studies (GWAS) in 2007. After GWAS studies conducted on people belong to different countries and ethnicities has discovered more than 80 susceptible loci connected to T2DM. These genes include KCNJ11, TCF7L2, IRS1, MTNR1B, PPARG2, IGF2BP2, CDKN2A, HHEX and FTO are found to correlated with development of T2DM. There are still a lot of unsorted loci responsible for pathogenesis of T2DM. To fully comprehend and manage T2DM, we must thus enhance our present biological understanding (Ali, 2013; Franks, 2012).

1.3.2 Environmental factors

Environmental risk factors leading to T2DM include age, dietary intake, fat distribution, obesity, sedentary lifestyle, genetic disposition, neglected prediabetes and gestational diabetes (shown in Figure 3).

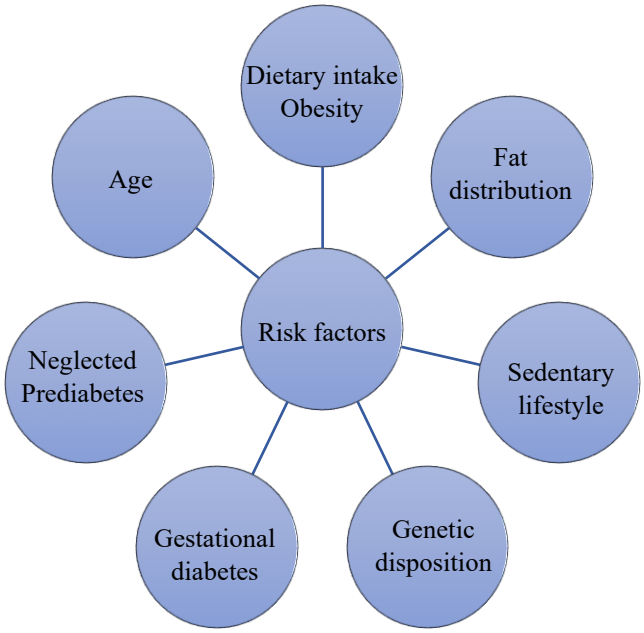

Figure 3: Risk Factors for T2DM (Bellou et al., 2018).

1.4 Diagnosis

Diagnosis criteria for diabetes is defined and updated by World Health Organization (WHO) and American Diabetes Association (ADA). Tests available for the diagnosis are mentioned below:

Fasting plasma glucose (FPG) test is used to measure the amount of glucose in blood in fasting state. Glucose in our body is tightly regulated by enzymes and metabolic reactions, any change from original value can lead to micro and macrovascular complications. Normal fasting glucose level is 80-100 mg/dL, level greater than 126 mg/dL indicates diabetes (Punthakee *et al.*, 2018).

Glycated haemoglobin (HbA1C) test indicates long term glycaemic control. This test is more concrete, as the result depicts the average glucose attached to haemoglobin in last 3 months (usual lifespan of RBCs). Normal level is < 5.7%, however, level > 6.5% are considered diabetic. Furthermore, Prediabetic patients show levels in between these two (5.7 – 6.5%) (Sherwani *et al*., 2016).

Oral glucose tolerance test (OGTT) is performed to check body's response to glucose. Blood is withdrawn before and after a liquid in high glucose concentration is given for oral uptake. Level of glucose before and after will explain efficiency of cells in taking up glucose. 140 mg/dL is considered normal, while concentration greater than 200mg/L is diabetic (Punthakee *et al.*, 2018; Ramachandra Bhat *et al*., 2019).

1.5 Treatments

For the treatment, combination therapy is usually preferred to treat diabetes, lifestyle adjustments and pharmaceutical treatment is provided to maintain glucose level in healthy range. Manufacturing cost, expected side effects, potential benefits, efficiency, shelf life and dosing schedule are all factors to consider before choosing a medicine. Insulin therapy is a widely used treatment option. Orally administered drugs like metformin, sulfonylureas,

thiazolidinediones and some inhibitors are also prescribed by physicians (Khan *et al*., 2019; Tan *et al*., 2019).

1.6 Significance of single nucleotide polymorphisms:

GLO1 gene is playing important role in maintain levels of toxic carbonyl moieties. According to Yin *et al* in 2021, functional SNP (inter genic SNP with reference ID rs1781735) of GLO1 gene is involve in the dysfunction of left middle frontal gyrus in case of schizophrenia. This shows anti-toxic role of GLO1 gene can be further studied to confirm its role in aetiology of schizophrenia and other neural defects (Yin *et al.,* 2021).

Another published study in 2022 tested two SNPs of GLO1 gene with rs4746 (C>A) and rs1130534 (A>C), and their association with Type II Diabetes Mellitus. They performed ARMS PCR and ELISA to study SNPs and MGO concentration respectively in disease and healthy patients. The results show these SNPs were associated with increase susceptibility to T2DM. ELISA test showed elevated levels of MGO in diabetic patients (Alhujaily *et al*., 2022).

In 2019, a study was conducted on *Coilia nasus* to analyse the effects of GLO1 polymorphisms on stress, antioxidation and inflammatory responses. Two SNPs from coding region were found to be cause changes in glo1 protein structure. Increased expression of functionally active glo1 protein was associated with rise in antioxidant and inflammatory responses. Ultimately it improves fish response to stress and reduces death rate (Du *et al.*, 2019).

1.7 Objectives of the study

Objectives of this research study are as follows:

- To study the functional role of regulatory non-coding SNPs of GLO1 gene by *in-silico* approach in T2DM.
- To investigate the association of GLO1 deleterious SNPs (rs141465532, rs753587598) with T2DM.

LITERATURE REVIEW

2.1 Type II Diabetes Mellitus and Methylglyoxal

Type II Diabetes Mellitus is marked by high glucose concentration in blood. During glycolysis methylglyoxal is produced. Methylglyoxal (MGO) is an α-oxoaldehyde naturally formed during sugar degradation or ingested from outside source (~3nmol/day). Endogenously, concentration of MGO is 50-300nM in blood plasma and 1000-2000uM in intracellular tissues (Kold-Christensen et al., 2019). MGO is an early glycation product including glyoxal and 3-deoxyglucosone. These α-oxoaldehydes are more reactive as compared to glucose and are involved in AGEs formation (Ogawa et al., 2010). Pathways depicting MGO production and AGEs formation are shown in figure 4.

2.1.1 Exogenous and endogenous source of methylglyoxal

Exogenously consumed food, beverages and pharmaceutical products like coffee, bread, biscuits, cookies, smoke and oils can cause elevation of MGO levels in body, as they contain micromolar concentration of MGO and can trigger pathways for endogenous formation (Wang & Chang, 2010; Zheng *et al*., 2021). Endogenously MGO is formed during glycolysis (triose phosphates fragmentation), glucose auto-oxidation, acetone metabolism involving ketone bodies and threonine catabolism (Frischmann *et al*., 2005; Zhang *et al*., 2023). Experiments revealed that MGO formation in plasma and tissue is mostly related to oral uptake of glucose, especially in oral glucose tolerance test (OGTT) (Zhang *et al.*, 2023).

2.2 Methylglyoxal and Type II Diabetes Mellitus

Methylglyoxal is a biomarker which is closely related to protein glycation and insulin resistance. MGO has been researched extensively as it can be a major player in T2DM development (Ramachandra Bhat *et al*., 2019). A study published by PJ Beisswenger in 2014, explained MGO as potential biomarker for diabetes as it possesses all needed potential traits.

(Beisswenger, 2014). MGO is considered as a dangerous di-carbonyl as it can disrupt normal body mechanisms like blood pressure. Exploring ways to reduce the concentration of MG in plasma can help us manage AGEs production and Type II Diabetes Mellitus incidence (Moraru *et al*., 2018; Ogawa *et al*., 2010)

2.3 Methylglyoxal related complications

Methylglyoxal is a toxic but inevitable compound formed naturally in our body. MGO can inhibit IRS-1/PI_3K pathway, this inhibition can decrease insulin secretion. It can also bind to glucose receptor GLUT4 and results in hyperglycaemia. MGO-Insulin adduct formation can disrupt insulin feedback mechanism. MGO is also harmful to adipose tissues leading to cell dysfunction (Ramachandra Bhat *et al*., 2019).

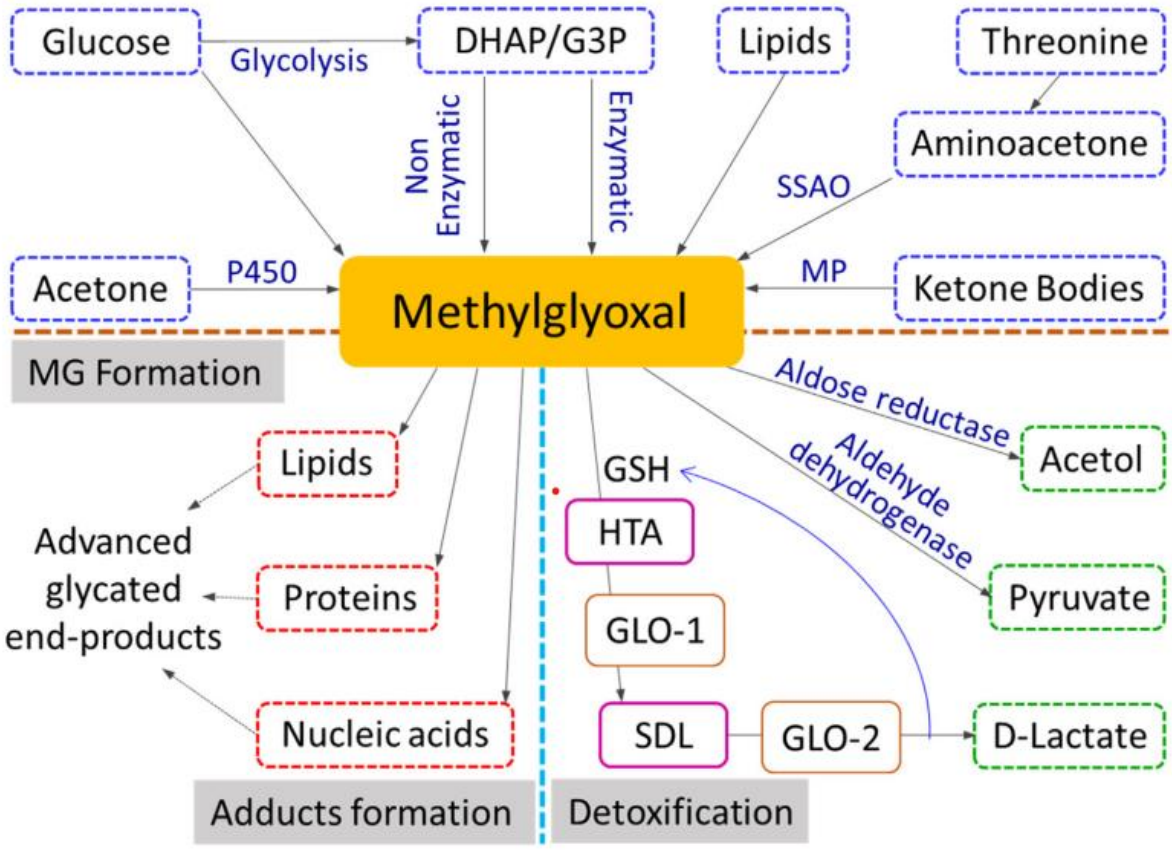

Figure 4: Pathways explaining MGO formation and reactions. (Ramachandra Bhat et al., 2019).

2.4 Methylglyoxal; precursors to Advance Glycation End Products

Reactive di-carbonyl molecules can react with amino and sulphydral groups in proteins (Cantero *et al*., 2007). Ex-vivo studies proved that AGEs formation in the presence of MGO is increased. MGO reacts preferably with arginine residues of proteins than lysine residues (Morgenstern *et al*., 2020). By introducing different concentrations of MGO in human plasma, scientists observed time and dose dependent formation of AGEs (Zhang *et al.*, 2023).

2.5 Advance Glycation End Products: Glycation and Millard reaction

In 1912, a French scientist Louis-Camille explains what happens when a sugar molecule is attached to a protein (glycation). The reaction was named as “Maillard reaction”. This reaction happens exogenously when we cook food, the browning in the process is due to Maillard reaction. The process was a subject of attention in coming years, until glycated haemoglobin was discovered in 1955. Glycated haemoglobin is a proof of endogenous glycation, now simply termed as Maillard reaction (*in-vivo*) (Fournet *et al*., 2018).

2.5.1 Advance Glycation End Products formation

Advanced glycation end products (AGEs) are irreversible products formed by a non-enzymatic reaction between sugar molecules to proteins and other macromolecules including DNA and lipids. Human body is naturally equipped with anti-glycation mechanism to remove or prevent AGEs formation, such as glyoxalase system. Abnormal functioning of these system can lead to AGEs accumulation. Under pathological or physiological conditions like diabetes, arthritis, cancer, hyperglycaemia or obesity aging, high level of AGEs is observed. However, reactive carbonyl species, such as methylglyoxal (MGO) and glyoxal (GO) are involved in glycation of proteins, nucleotides and phospholipids and can cause significant damage to proteome, genome and lipidome (Morgenstern *et al*., 2020; Saeed *et al*., 2020).

2.5.2 Advance Glycation End Products related complications

Reactive α-oxoaldehyde metabolites and associated AGEs can be harmful in many ways. Some with the examples are shown in Figure 5.

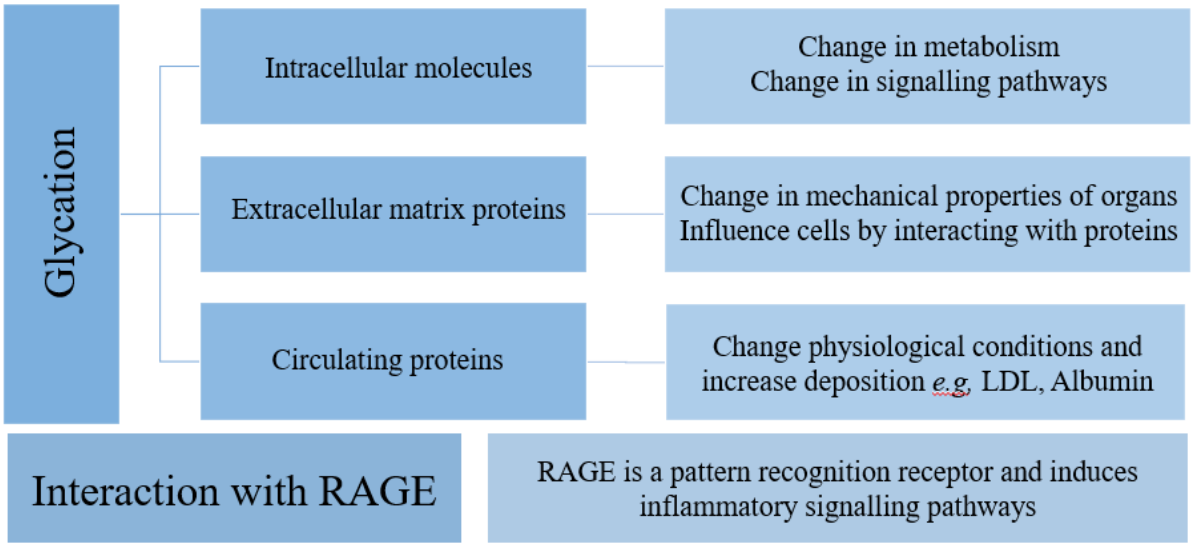

Figure 5: AGEs interaction with proteins and possible damage (Wortmann et al., 2014).

2.6 Advance Glycation End Products and diabetes

Advance Glycatrion End Products (AGEs) are found 5-15 times more in diabetic patients than normal when compared to other biomarkers (Beisswenger, 2014; Brings *et al.*, 2017). AGEs precursors and oxidative stress are found to be associated with diabetes, they are studied to explore other metabolic pathways related to incidence of diabetes (Moraru *et al.*, 2018).

2.6.1 Advance Glycation End Products involvement in wound healing

In 2007, Cantero and group published their research on the linkage between AGEs precursors and PDGFRβ receptors. They find AGE-PDGFRβ adducts in mesenchymal, SMC and fibroblasts when treated with GO and MGO. These adducts were discovered in diabetic mice model rendering PDGFRβ dysfunctional, leading to reduce tissue repair and wound healing in patients suffering with diabetes (Cantero *et al.*, 2007).

2.7 Glyoxalase system

Glyoxalase system is a primary innate mechanism to detoxify reactive aldehydes formed during carbohydrate (hyperglycaemic) metabolism. It is a highly conserved system with cooperating enzymes existing in simple prokaryotes to complex mammalian organisms (Morgenstern *et al*., 2020). Major substrate for this reaction is MG. With this mechanism, MGO is converted to less reactive D-Lactic acid in a two-step reaction with the help of two intra-cellular enzymes; glyoxalase I (Glo1) and glyoxalase II (Glo2). Glyoxalase I enzyme (lactoylglutathione lyase) is a ubiquitously expressed enzyme catalysing the conversion of MGO to S-D-Lactoylglutathione (hemithioacetal) with the help of catalyst glutathione (GSH) (Figure 6). It is a rate limiting step in glyoxalase system, making it significantly important in maintaining levels of glycated products or AGEs (He *et al.*, 2020; Yumnam *et al*., 2021).

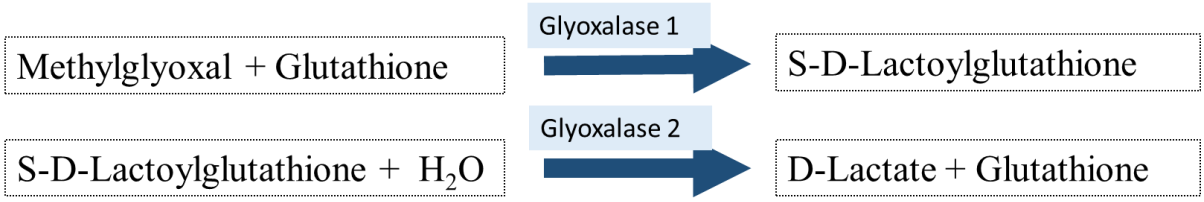

Figure 6: Systematic representation of glyoxalase system.

2.7.1 Glyoxalase 1 gene

2.7.1.1 Nomenclature

Glyoxalase 1 or GLO1 is a gene encoding glyoxalase I enzyme (lactoylglutathione lyase). Other aliases for this gene are GLYI, GLOD1 and HEL-S-74) (Farrera & Galligan, 2022).

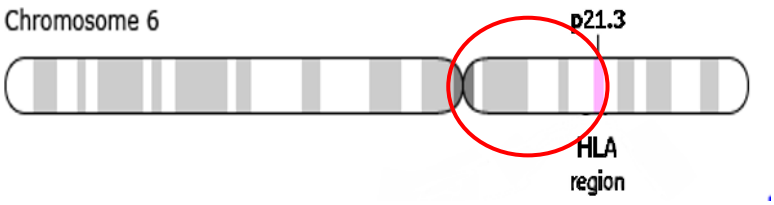

Figure 7: Schematic presentation of Glyoxalase 1 gene.

2.7.1.2 Location

GLO1 gene is a protein coding gene located on 6p21.2. It is flanked by Human Leukocyte Antigen HLA and centromere of chromosome 6 on the short arm of chromosome (Figure 7). It is linked to HLA and transcribes glo1 enzyme, a notably important protein (Farrera & Galligan, 2022).

2.7.1.3 Structure

GLO1 gene is approximately 27000 bp long. GLO1 contains 6 exons and 5 introns. GLO1 gene is orthologous with 425 other organisms. Many species have conserved regions for this gene, homolog for GLO1 are found in chimpanzee, Rhesus monkey, dog, cow, fruit fly, *A. thaliana* etc (Ensembl release 109). It is ubiquitously expressed in prostate and duodenum and 24 other tissues.

2.7.1.4 Isoforms encoded

GLO1 gene has two splice variants or transcripts. Among them, only one is protein coding.

Table 1: Protein coding transcripts and isoforms from GLO1 gene.

Transcript ID	Transcripts name	Nucleotide number	Protein	Biotype	UniProt match
ENST00000373365.5	GLO1-201	2016	184aa	Protein coding	Q04760-1
ENST00000470973.1	GLO1-202	1939	No protein	Protein coding CDS not defined	-

2.7.2 Glyoxalase I enzyme

Glyoxalase 1 enzyme or lactoylglutathione lyase is a 184 amino acid long proteinaceous enzyme transcribed from chromosome 6. Glo1 is a metal-containing enzyme, its core can be made of zinc (humans, yeast, *E. coli*) or nickel (*Trypnosoma cruzi, L.major*). Regulation of glo1 enzyme involves threonine (by phosphorylation) and cysteine residues (modification mediated by nitric-oxide) (Saeed *et al*., 2020). Glo1 enzyme is mainly involve in maintaining low levels of MG in plasma. It is an important part of glyoxalase system due to its involvement in rate-limiting step. Glo1 enzymes makes 10% of the total plasma protein concentration in all eukaryotic cells (Morgenstern *et al*., 2020).

2.7.2.1 Significant role of Glyoxalase 1 enzyme

Glyoxalase 1 enzyme has a complex regulation, and many areas can be explored related to its pathway. However, it contains many responsive elements for, antioxidant, metal, and insulin. It has high activity for copy number variations. Upregulation (by Nef2 elevation) and downregulation (by hyperglycaemia and hypoxia) of Glo1 enzyme is directly linked with MGO homeostasis in cytosol. Many studies suggest that Glo1 impact cell morphology of healthy cells in stress condition and cancerous cells in increased energy demand (Morgenstern *et al*., 2020). Research done on *Caenorhabditis elegans* by Morocos et al in 2008 suggest that increase Glo1 activity will reduce MG level resulting in low ROS production. This experiment resulted in increased life span by 40% (Morcos *et al.,* 2008). In hyperglycaemic conditions, overexpression of glo1 enzyme reduces AGEs accumulation in endothelial cells and reduce ateriogenesis. Furthermore, haperglycaemia and diabetic complications related to nervous or cardiovascular system are related abnormal functioning of Glo1 and elevated levels of MG (Morgenstern *et al.*, 2020; Saeed *et al*., 2020). However, induction of Glo1 by hesperidin gave positive health benefits to patients suffering from obesity, cardiovascular and diabetic disease.

MATERIALS AND METHODS

3.1 *In-Silico* Analysis of Regulatory Single Nucleotide Polymorphisms

3.1.1 Data mining for regulatory SNPs

The SNP data (reference IDs) on Human gene Glyoxalase 1 (GLO1) was retrieve from Ensemble database (https://asia.ensembl.org; accessed March 2023). After searching human gene GLO1 on Ensembl, we get SNPs, consequences for intronic, 3' and 5' variants were turned on and downloaded as a single file. Obtained SNPs were screened for minor allele frequency equal and above 0.01 (MAF ≥ 0.01) to get a final dataset (as shown in figure 2). To substantiate the data, SNPs for GLO1 gene from dbSNP database (https://www.ncbi.nlm.nih.gov; accessed

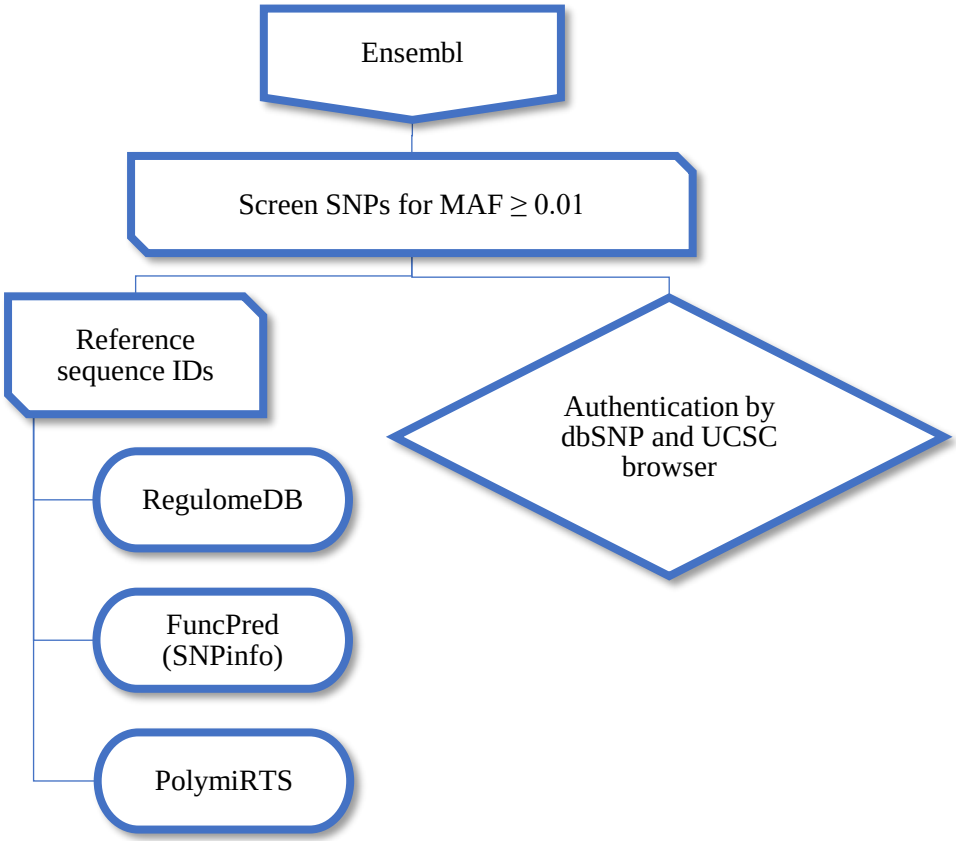

Figure 8: Schematic diagram for in-silico analysis of GLO1 gene by computational tool.

on May 2023) and UCSC genome browser (https://genome.ucsc.edu; accessed on May 2023) were also extracted and corroborated with selected dataset (from Ensembl). Results showed that among 111 Ensembl extracted SNPs, 107 were reported in both dbSNP and UCSC genome

browser (Table 3). The strategy followed for computational analysis of Glo1 SNPs is given in figure 8.

3.1.2 Analysing molecular effects of regulatory SNPs

Intronic, 5 prime and 3 prime SNPs were investigated by using three different tools to analyse their regulatory function.

RegulomeDB is an integrated platform containing data on functional aspect of non-coding DNA polymorphism. Data on RegulomeDB comes by intersecting position of polymorphisms

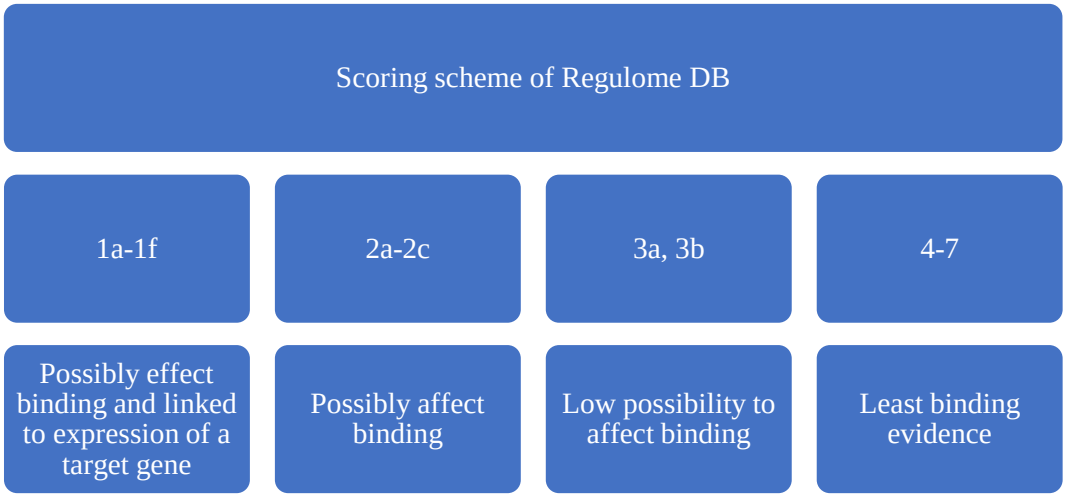

Figure 9: Annotation scores of RegulomeDB with consequences.

with functionally active non-coding regions. Information on functionally active non-coding regions was obtained from genomic assays (TF Chip-seq and DNase-seq) which provides computational outputs (https://regulomedb.org; accessed May 2023). RegulomeDB provide scored and prioritised results of variants which helps in identifying variants with more significant regulatory role shown in figure 9 (Boyle *et al.*, 2012). Score annotations of variants on database range from 1-7, 1 being most functionally significant while 6 being least. 7 is for SNPs having no annotation data. Reference IDs of filtered SNPs were submitted in Regulome DB.

FuncPred (SNP function prediction) is a discreate tool of SNPinfo server. FuncPred is used to predict polymorphisms which changes TFBS (transcription factor binding site), ESE (Exonic

splicing enhancers) and miRNA binding sites (https://snpinfo.gov; accessed March 2023). We used this tool to predict functional aspects of variants under study (Xu and Taylor., 2009). The selected DNA polymorphisms were queried based on their reference IDs.

PolymiRTS (Polymorphisms in microRNA and Target Sites,) is an unconfined online database. It is used to investigate DNA polymorphisms related to miRNA (https://polymiRTS.edu; accessed May 2023). It provides on DNA variants disrupting miRNA-mRNA interactions, miRNA target sites, miRNA seed regions and biological pathways (Bhattacharya *et al*., 2014). "GLO1" was searched in 'gene search' portion of PolymiRTS. Lists of SNPs for different categories were retrieved.

3.2 *IN-VITRO* ANALYSIS OF SNPs (rs141465532 and rs753587598)

Genetic association study for SNPs was done on the missense SNPs of GLO1 gene. This study was approved by Institutional Review Board (IRB) of National University of Science and Technology (NUST).

3.2.1 Sample size and blood collection

In the present association study, SNPs of GLO1 gene were studied for their significant role in T2DM patients in comparison to healthy patients. A total of 150 blood samples were obtained, including 50 healthy and 100 T2DM patients (Figure 10). Sample blood collection was performed after taking informed consent from donor. Patients were asked about their family history and other complication they might have, like cardiovascular diseases. The samples were taken from collaborating hospitals over the period of 3 to 4 months.

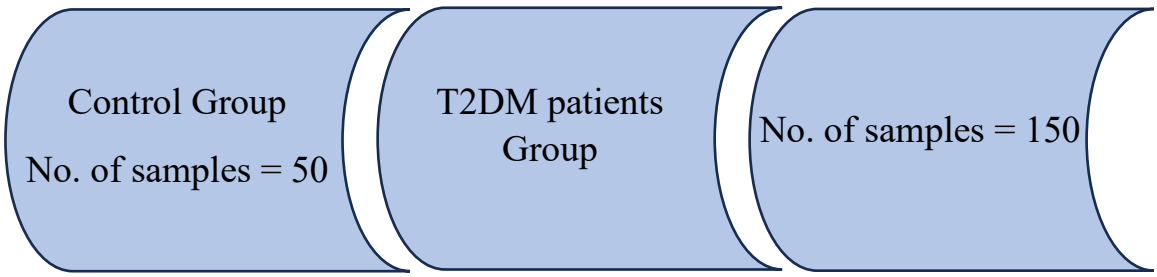

Figure 10: Distribution of sample size.

Blood samples were taken with the help of nursing staff. Syringes of 5cc (ml) or 10cc (ml) were used to collect blood in vacutainer tubes (EDTA vials). Vials are labelled with name, age, and gender. Collected samples are stored at 4^{o} C in lab.

3.2.2 Variants selection of Glyoxalase 1 gene

Genetic variants of GLO1 gene with reference sequence IDs rs141465532, rs753587598 and rs1038747749 were selected for *in-vitro* analysis on blood samples of T2DM patients. These SNPs were shortlisted previously by our research group with the help of computational tools. SNPs with consequence, amino acid change and alleles are shown in table 2.

3.2.3 Organic method of DNA Extraction and Purification

DNA from blood samples was extracted by phenol-chloroform method. It is an organic method of extraction. Blood of volume 750ul was added to 1.5ml micro centrifuge tube (Axygen, California, USA). 750ul volume of A solutrion is added to same tube and is incubated at room temperature for 5-10 min. Centrifugation was performed at 13000rpm for a minute. Supernatant was removed and resuspension of pallet was done by adding solution B (400ul), 20$ SDS (12ul), 5ul of proteinase K solution. Solution was left overnight on room temperature.

Equal ratio of solution C and D (500ul each) was mixed and added to previous tube. Centrifugation for 10 minutes was done at 13000 rpm. Upper aqueous layer is separated with great care into new tube followed by the addition of 55ul CH_3COONa and isopropanol in equal ratios. Inversion of tube for multiple times was performed and again centrifugation was done at 13000rpm to separate DNA. DNA pallet obtained after removing supernatant was subjected to 70% chilled ethanol, it was left to dry at room temperature. Following the evaporation of ethanol DNA was dissolved in Tris-EDTA.

3.2.3.1 Reagents preparation

All glass wear and distilled water were autoclaved before making solution. Triton-X was added after autoclaving solution A. Solution A is used for the lysis of blood cells. 1L is prepared by adding 109.44g Sucrose (0.32M), 1.21g 10mM Tris (pH 7.5), 1.01g 5mM MgCl2, 1% (v/v) Triton X-100. Solution B works in precipitation of DNA and proteins. 1L is prepared by adding 1.21g 10mM Tris (pH 7.5), 23.37g 400mM NaCl, 0.58g 2mM EDTA (pH 8.0). Solution C is phenol. It is used for DNA isolation. Solution D is prepared by adding chloroform and isoamyl alcohol in 1:1. It is used to purify DNA. 20% SDS solution is prepared by adding 20g in 100ml of distilled water.

3.2.4 1% Agarose gel electrophoresis

To verify the presence and quality of extracted DNA, we prepare 1% agarose gel. Agarose gel electrophoresis can efficiently separate DNA which was visualized in UV transilluminator and gel doc system.

3.2.4.1 Gel preparation and electrophoresis

Gel was prepared by dissolving 1g agarose in 1X TAE buffer, heated in oven, to form 100ml solution. This gel solution was cooled until evaporation stopped; 3µl ethidium bromide is added to the solution. Gel solution was poured in gel-casting tray and was left to solidify. Afterwards, gel was loaded with samples mixed with loading dye and was placed in buffer tank. Buffer tank was filled with 1X TAE buffer. 1L 1X TAE buffer was made by adding 20ml 50X TAE buffer [242g Tris base, 57.1ml glacial acetic acid and 100ml of 500mM EDTA solution (pH=8.0) are added, and volume is raised to 1litre] in 980ml of distilled water.

3.2.5 DNA Quantification and dilutions

The isolated genomic DNA was quantified with the help of microvolume spectrometer LB 915 Colibri (NanoDrop). At first, the PCR water was used for blank and then the sample was loaded

to calculate the absorbance ratio. The absorbance of nucleic acids was arbitrated at wavelength 260nm optimum absorption was recorded. The absorbance ratio of 260/280nm was suggestive towards purity of DNA (1.8 - 2.2 is considered "pure" for nucleic acid).

3.2.6 Primer designing

To perform High resolution melting analysis (HRM), one forward and one reverse primer was designed for SNPs utilizing tools such as dbSNP, Ensemble, Primer 3, Europhin, Oligocalc and UCSC genome explorer.

Table 2: Deleterious SNPs description with primer sequences.

rsID	Amino acid change	Allele	Consequence	Primer Name	Primer Sequence (5' to 3')
rs141465532	Ile128Thr	(A>G)	Missense Variant	GLO132FW	CATCAGGAACAGCAATTCCAA
				GLO132RC	CTTCTGCCTTGATCTCCAGTC
rs753587598	Phe125Cys	(A>C)	Missense Variant	GLO198FW	AAACAGGCAAACTTACCGA
				GLO198RC	GTTCTTGTCCAGTACCTGTAT

FASTA sequence for SNPs was retrieved by entering SNP ID in dbSNP, a database for identifying gene variations. Hair-pin formation and self-complementarity were verified by entering that SNP sequence in Oligocalc, a software for calculating oligonucleotides. Subsequently, *in-silico* PCR in reference to UCSC genome browser was done to verify the specific binding of primers through target allele, as it calculates the hypothetical effects of PCR and gets to know the amplicon size. Forward and reverse primers were entered to amplify the target DNA sequence, and computational amplification was tested.

3.2.7 High Resolution Melt Curve Profiling

We performed high resolution melting (HRM) analysis on SNP with rsID rs1038747749 to amplify a DNA fragment of length of 207 nucleotides with primers GLO149RW (5'TGCAGCAGACCATGCTACG3') and GLO149FC(5'GCCTAATCACAGACTCCTG3').

The reaction mix of 20µl was prepared by adding 10µl PCR water, 1µl reverse primer, 1µl forward primer, 2µl template DNA and 8µl Cyber green qPCR master mix.

All HRM-PCR reactions were performed on AB QuantGene automated real time PCR system (SYSTAAQ Diagnostic Products). The HRM-PCR protocol consists of initial preincubation at 95^{o}C for 12:00, then 40 cycles for 30s at 95^{o}C, 45s at 61^{o}C, 30s at 72^{o}C. Finally, melt analysis was performed at 2^{o}C / s.

3.2.8 Statistical Analysis

The results were statistically analysed using "GRAPHPAD PRISM 10" and online 2 X 2 contingency table. Fisher's exact test, Odds ratio, Relative risk, Genotypic frequency & Allelic frequency were calculated. P values; *P: <0.05, **P: <0.01, ***P: <0.001 were considered as significant.

RESULTS

4.1 *In-silico* Analysis

4.1.1 SNP retrieval dataset

Human gene "GLO1" was searched on Ensembl, and we extracted a total of 5201 SNPs; including 4921 intronic, 237 3' UTR and 43 5' SNPs from Ensembl (Ensembl Release 109; accessed March 2023). after screening 5201 SNPs for MAF ≥ 0.01, we got a total of 111 SNPs, as shown in figure 2. The reference sequence IDs of these 111 SNPs were tallied from dbSNP-NCBI (https://www.ncbi.nlm.nih.gov; accessed on May 2023) and UCSC (https://genome.ucsc.edu; accessed on May 2023) to get a final dataset of 107 SNPs (Table 3).

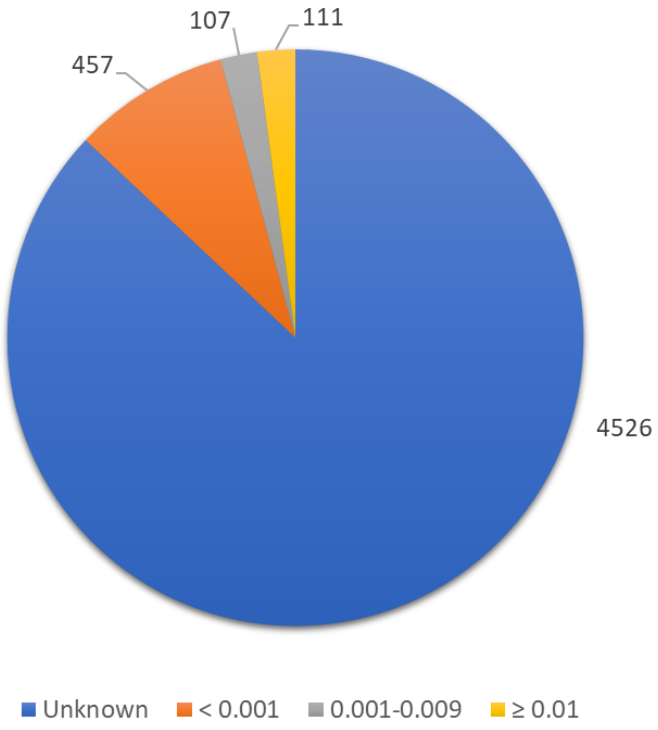

Figure 11:All SNPs with minor allele frequencies from Ensembl.

Table 3: SNPs screened from three online data bases.

Variant ID	Alleles	Global MAF	Class	Source	Consequence Type
rs16890915	A/G	0.015	SNP	dbSNP	3’ UTR variant
rs75200860	T/A	0.016	SNP	dbSNP	3’ UTR variant
rs7604	C/T	0.052	SNP	dbSNP	3’ UTR variant
rs9470916	C/A	0.129	SNP	dbSNP	3’ UTR variant
rs1049346	G/A/C	0.435	SNP	dbSNP	5’ UTR variant
rs10447398	G/A/C	0.237	SNP	dbSNP	intron variant
rs10484854	C/T	0.102	SNP	dbSNP	intron variant
rs10947755	T/C	0.463	SNP	dbSNP	intron variant
rs111345107	C/T	0.126	SNP	dbSNP	intron variant
rs111624192	G/A/C/T	0.044	SNP	dbSNP	intron variant
rs112436015	G/A	0.014	SNP	dbSNP	intron variant
rs114031410	C/T	0.02	SNP	dbSNP	intron variant
rs114153557	G/A	0.02	SNP	dbSNP	intron variant
rs114637114	G/A	0.02	SNP	dbSNP	intron variant
rs114762451	A/G	0.046	SNP	dbSNP	intron variant
rs115199318	A/C	0.014	SNP	dbSNP	intron variant
rs115962292	A/G	0.015	SNP	dbSNP	intron variant
rs116697025	A/G	0.015	SNP	dbSNP	intron variant
rs117588402	T/C	0.021	SNP	dbSNP	intron variant
rs117707770	C/T	0.02	SNP	dbSNP	intron variant
rs12209475	C/T	0.192	SNP	dbSNP	intron variant
rs12209477	C/G/T	0.187	SNP	dbSNP	intron variant
rs12210866	C/T	0.187	SNP	dbSNP	intron variant
rs12212511	G/A	0.354	SNP	dbSNP	intron variant
rs12214815	G/C/T	0.182	SNP	dbSNP	intron variant
rs12526818	G/T	0.051	SNP	dbSNP	intron variant
rs13196356	G/A	0.104	SNP	dbSNP	intron variant
rs13200763	C/A/G	0.187	SNP	dbSNP	intron variant
rs13212218	G/A/C	0.052	SNP	dbSNP	intron variant
rs13215896	T/A/C	0.05	SNP	dbSNP	intron variant
rs13216215	C/T	0.111	SNP	dbSNP	intron variant
rs13219781	G/A	0.05	SNP	dbSNP	intron variant
rs140084648	G/A	0.052	SNP	dbSNP	intron variant
rs140458850	C/T	0.046	SNP	dbSNP	intron variant
rs147563311	G/A	0.021	SNP	dbSNP	intron variant
rs148519062	T/A	0.015	SNP	dbSNP	intron variant
rs148831289	T/C	0.013	SNP	dbSNP	intron variant
rs150649525	C/G/T	0.049	SNP	dbSNP	intron variant
rs1579028	G/A	0.173	SNP	dbSNP	intron variant
rs1621483	G/A/T	0.206	SNP	dbSNP	intron variant
rs1623947	T/A/C	0.409	SNP	dbSNP	intron variant
rs1626200	A/C/T	0.338	SNP	dbSNP	intron variant
rs1626217	A/G	0.167	SNP	dbSNP	intron variant
rs16890922	C/T	0.024	SNP	dbSNP	intron variant
rs1698987	G/A	0.033	SNP	dbSNP	intron variant

rs17622621	G/A	0.188	SNP	dbSNP	intron variant
rs1781715	A/G/T	0.244	SNP	dbSNP	intron variant
rs1781716	G/C	0.123	SNP	dbSNP	intron variant
rs1781717	C/G/T	0.418	SNP	dbSNP	intron variant
rs1781719	C/A/T	0.418	SNP	dbSNP	intron variant
rs1781737	T/A/G	0.31	SNP	dbSNP	intron variant
rs2277109	C/A/G	0.052	SNP	dbSNP	intron variant
rs2455773	G/A/T	0.123	SNP	dbSNP	intron variant
rs2471999	G/T	0.446	SNP	dbSNP	intron variant
rs2490026	C/G/T	0.499	SNP	dbSNP	intron variant
rs2736655	A/C/G/T	0.145	SNP	dbSNP	intron variant
rs2894415	T/A/C	0.068	SNP	dbSNP	intron variant
rs2894416	G/C	0.068	SNP	dbSNP	intron variant
rs34349982	G/A	0.054	SNP	dbSNP	intron variant
rs34637217	C/T	0.05	SNP	dbSNP	intron variant
rs34971977	T/C	0.053	SNP	dbSNP	intron variant
rs35207097	G/A	0.053	SNP	dbSNP	intron variant
rs375414591	A/G	0.059	SNP	dbSNP	intron variant
rs3778443	G/A/T	0.07	SNP	dbSNP	intron variant
rs3799703	A/G	0.414	SNP	dbSNP	intron variant
rs4711556	T/C	0.173	SNP	dbSNP	intron variant
rs4714175	A/G	0.442	SNP	dbSNP	intron variant
rs55784543	G/A	0.102	SNP	dbSNP	intron variant
rs57156564	G/A	0.183	SNP	dbSNP	intron variant
rs57204119	G/C	0.044	SNP	dbSNP	intron variant
rs58702853	A/G	0.014	SNP	dbSNP	intron variant
rs58749921	A/G	0.235	SNP	dbSNP	intron variant
rs59905759	C/T	0.014	SNP	dbSNP	intron variant
rs60262339	G/C	0.173	SNP	dbSNP	intron variant
rs60896390	C/T	0.014	SNP	dbSNP	intron variant
rs61287418	C/T	0.016	SNP	dbSNP	intron variant
rs62396384	G/A	0.068	SNP	dbSNP	intron variant
rs62396385	G/C	0.07	SNP	dbSNP	intron variant
rs6932648	C/T	0.231	SNP	dbSNP	intron variant
rs6935120	C/T	0.015	SNP	dbSNP	intron variant
rs71571340	A/G	0.011	SNP	dbSNP	intron variant
rs71571342	G/C	0.011	SNP	dbSNP	intron variant
rs72856546	G/T	0.109	SNP	dbSNP	intron variant
rs73414381	G/A	0.014	SNP	dbSNP	intron variant
rs73414382	G/A/T	0.014	SNP	dbSNP	intron variant
rs73414392	T/G	0.014	SNP	dbSNP	intron variant
rs73414393	C/T	0.014	SNP	dbSNP	intron variant
rs73414402	G/A/C	0.034	SNP	dbSNP	intron variant
rs73734417	A/G	0.075	SNP	dbSNP	intron variant
rs74423628	A/G	0.05	SNP	dbSNP	intron variant
rs74721526	C/T	0.02	SNP	dbSNP	intron variant
rs75203003	A/G	0.015	SNP	dbSNP	intron variant
rs75990857	A/G	0.021	SNP	dbSNP	intron variant

rs76070225	A/T	0.019	SNP	dbSNP	intron variant
rs76395406	G/A	0.015	SNP	dbSNP	intron variant
rs7761715	T/C	0.014	SNP	dbSNP	intron variant
rs79023646	C/T	0.053	SNP	dbSNP	intron variant
rs9366973	A/G/T	0.443	SNP	dbSNP	intron variant
rs9369075	C/A	0.246	SNP	dbSNP	intron variant
rs937662	C/T	0.355	SNP	dbSNP	intron variant
rs9394522	G/A	0.163	SNP	dbSNP	intron variant
rs9394523	C/T	0.246	SNP	dbSNP	intron variant
rs9394524	T/G	0.249	SNP	dbSNP	intron variant
rs9462450	T/C	0.282	SNP	dbSNP	intron variant
rs9470917	A/C	0.175	SNP	dbSNP	intron variant
rs9470918	C/A/T	0.175	SNP	dbSNP	intron variant
rs9791292	G/A	0.123	SNP	dbSNP	intron variant

Regulome DB

Reference sequence IDs of 107 SNPs were submitted to RegulomeDB. Output data for 107 SNPs was obtained. 41 SNPs were found with annotation score range from 1 - 3, 51 SNPs with annotation score from 4 - 6, while remaining 15 SNPs fall in the last category with score 7 (Figure 10).

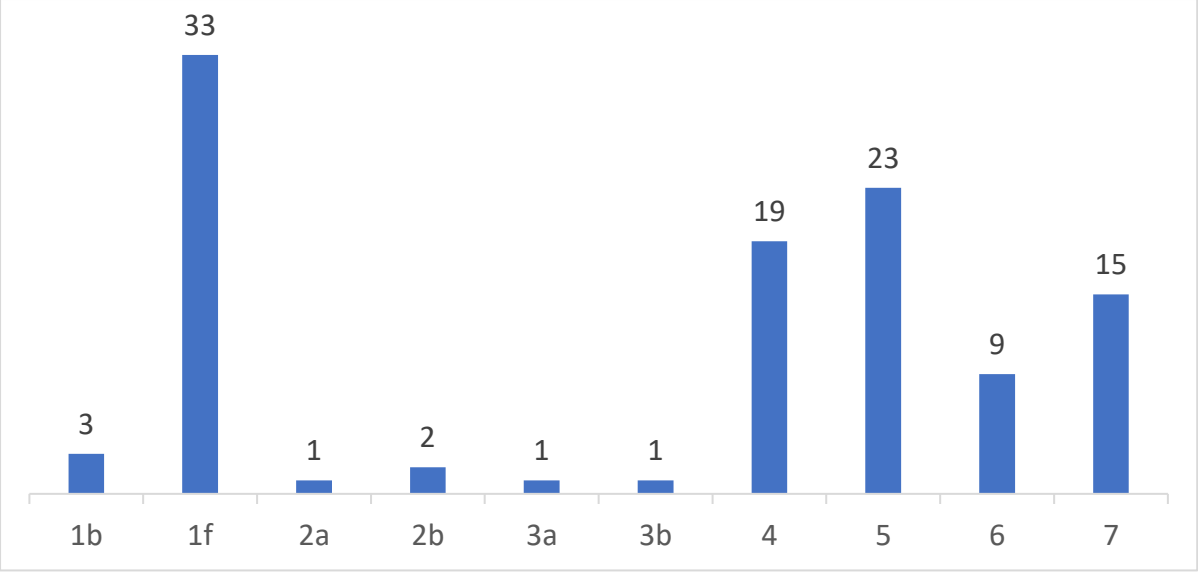

Figure 12: Distribution of annotation scores from RegulomeDB.

FuncPred

SNP Function prediction result was obtained for 71 SNPs. In the obtained results, 3 SNPs (rs1049346, rs2277109, rs2490026) were predicted to lie in TFBS (transcription factor binding site) and 3 more (rs16890915, rs7604, rs9470916) were predicted to affect miRNA binding (Table 4).

Table 4: List of non-coding SNPs obtained from FuncPred.

SNPs	TFBS	miRNA
rs1049346	✓	
rs16890915		✓
rs2277109	✓	
rs2490026	✓	
rs7604		✓
rs9470916		✓

PolymiRTS

To understand the potential effect of GLO1 polymorphisms on the miRNA functioning and target sites, we screened SNPs from PolymiRTS. A total of 18 3' SNPs were retrieved. Among the 3' SNPs retrieved, 4 SNPs (rs7604, rs9470916, rs75200860, rs16890915) are from our selected data set (Table 5).

Table 5: List of 3' SNPs screened from PolymiRTS..

rsIDs	Alleles	miRNA motif	Functional class	Context + score
rs7604	G	hsa-miR-500b-3p hsa-miR-5096 hsa-miR-6802-3p	D D D	-0.203 -0.15 -0.217
	A	hsa-miR-500b-3p hsa-miR-5096 hsa-miR-6802-3p hsa-miR-500b-3p hsa-miR-5096 hsa-miR-6802-3p	C C C C C C	0.053 -0.096 -0.096 -0.105 -0.155 -0.161
rs9470916	T	hsa-miR-338-5p	C	0.055
rs75200860	A	hsa-miR-548c-3p	D	0.07
rs16890915	T	hsa-miR-4724-5p	D	-0.198
	C	hsa-miR-4468 hsa-miR-6857-3p	C C	-0.149 -0.21

4.2 *In-vitro* Analysis

High resolution analysis was performed was performed to ascertain the association of GLO-1 gene polymorphisms with susceptibility of Type II Diabetes Mellitus. We

4.2.1 DNA Extraction and Quantification

DNA form blood samples was extracted in two days by using organic (phenol-chloroform) method. NanoDropTM 2000 software was used to assess the quality and quantity of extracted genomic DNA. The 260/280 ratio was established to ensure genomic DNA purification.

4.2.2 Analysis of GLO-1 rs1038747749 polymorphism

Analysis of GLO-1 polymorphism with rs1038747749 was done by performing HRM analysis to get melt curves. Blood samples were screened to confirm the presence or absence of specific allele. Melt curves and peaks were obtained for the samples. Melt peaks for blood samples of controls and patients are shown in Figures 13 and 14.

GLO-1 rs1038747749 is an SNP variant present on second exon at chromosome 6 (6:38686946). For this SNP, nucleotide C>T variation occurs where C is ancestral allele and T

is risk allele. Consequence of this SNP is the change in amino acid at position 38 from arginine to glutamine i.e., Arg38Gln.

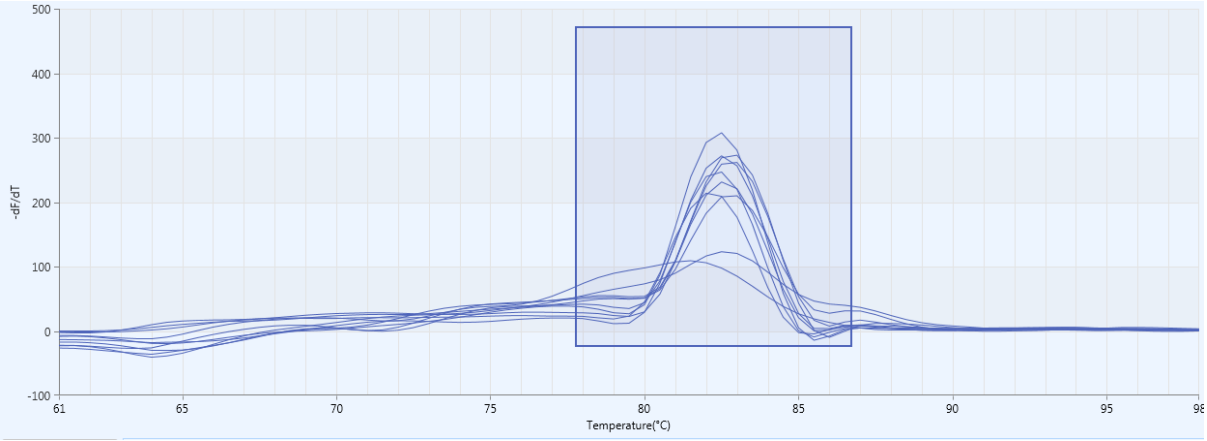

Figure 13: Melt peaks obtained from HRM analysis of control group for GLO-1 rs1038747749.

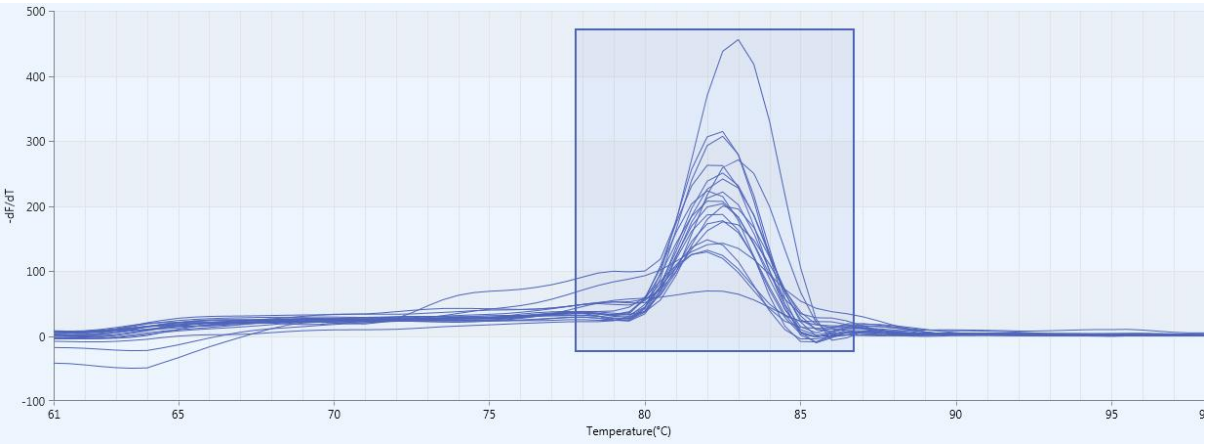

Figure 14: Melt peaks obtained from HRM analysis of patient samples for GLO1 rs1038747749.

4.2.3 Association GLO-1 rs1038747749 polymorphism with T2DM susceptibility

GLO-1 rs1038747749 showed a significant association with T2DM. Calculated p-value is 0.486*, which confirms the significant association of rs1038747749 with disease prevalence. Genotypic frequencies with respect to the presence and absence of ancestral and risk allele are shown in figure 15. It shows more presence of mutant variant in Diabetic patients.

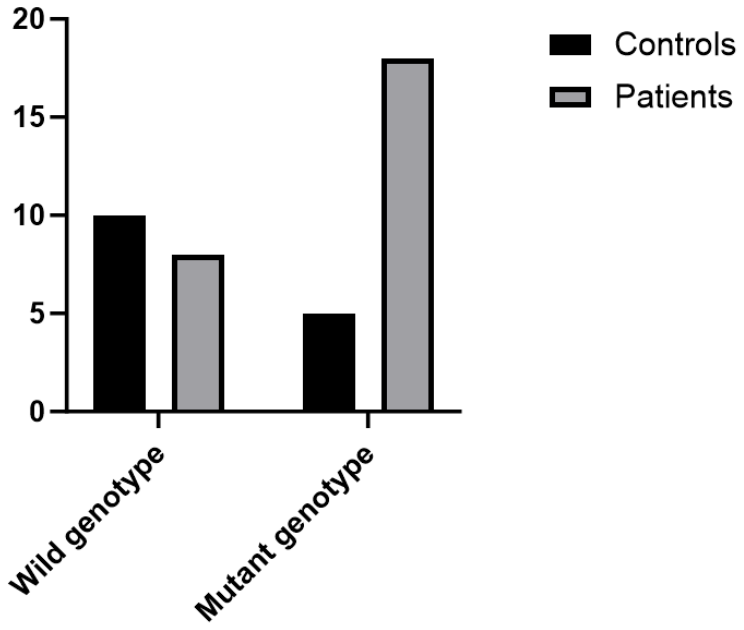

Figure 15: Variant genotype distribution of GLO-1 rs1038747749 in healthy and diabetic patients.

4.2.4 Analysis of GLO-1 rs753587598 polymorphism

Analysis of GLO-1 polymorphism with rs753587598 was done by performing HRM analysis to get melt curves. Blood samples were screened to confirm the presence or absence of specific allele. Melt curves and peaks were obtained for the samples. Melting peaks are shown in figure 16 and 17.

GLO-1 rs753587598 is an SNP variant present on forth exon at chromosome 6 (6:38682810). For this SNP, nucleotide A>C variation occurs, where A is ancestral allele and C is a risk allele. Consequence of this SNP is the change in amino acid at position 125 from phenylalanine to cysteine i.e., Phe125Cys.

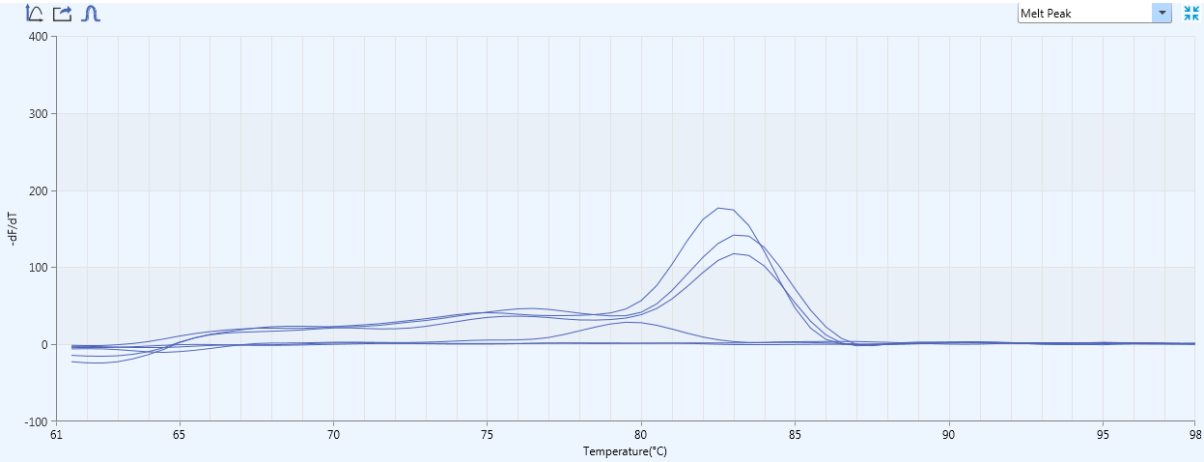

Figure 16: Melt peaks obtained from HRM analysis of control group for GLO1 rs743587598.

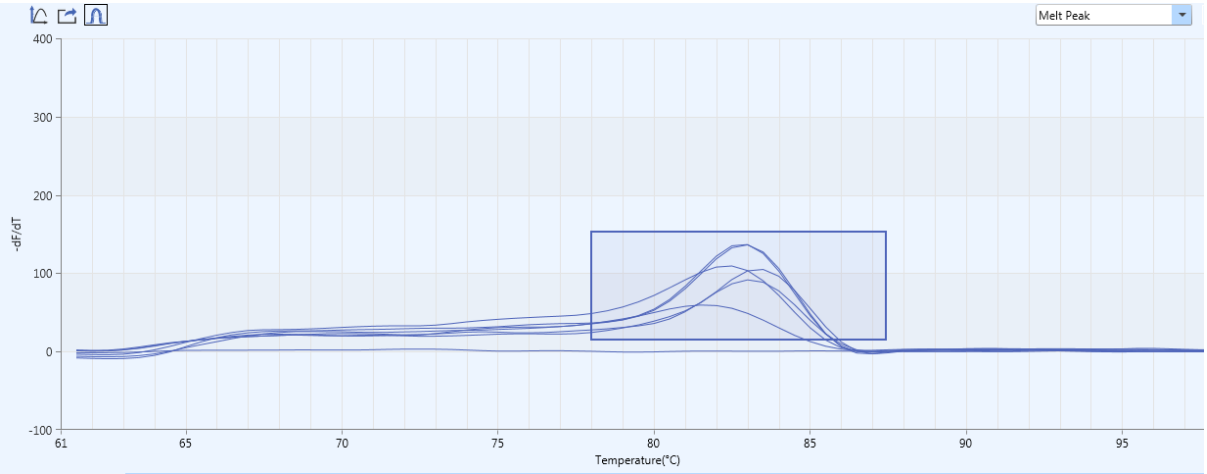

Figure 17: Melt peaks obtained from HRM analysis of patient group for GLO1 rs743587598.

4.2.5 Association GLO-1 rs753587598 polymorphism with T2DM susceptibility

GLO-1 rs753587598 showed no association with T2DM. Calculated p-value is >0.05, which confirm no significant association of this SNP with T2DM. Genotypic frequencies with respect to the presence and absence of ancestral and risk allele are shown in figure 18.

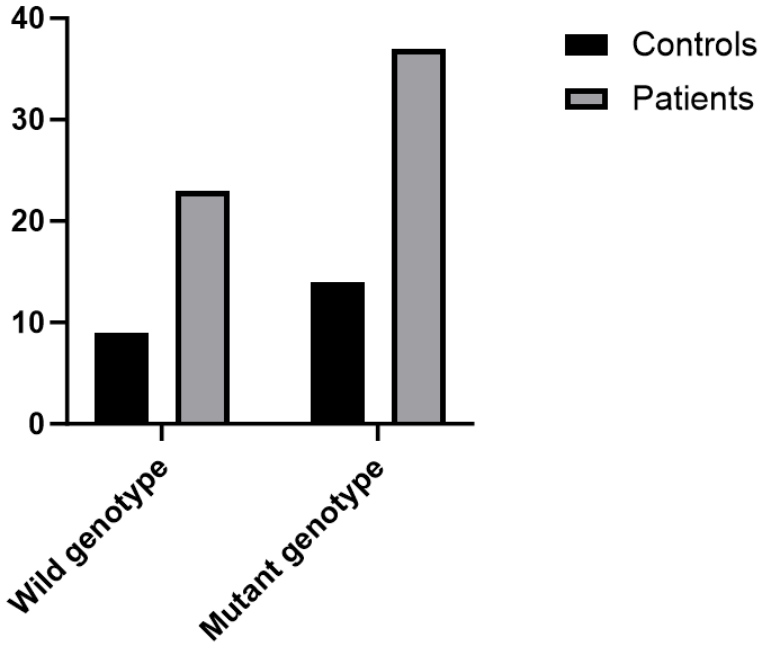

Figure 18:Variant genotype distribution of GLO-1 rs753587598 in healthy and diabetic patients.

DISCUSSION

Type II Diabetes Mellitus (T2DM) is a non-communicable disease of global concern. Glyoxalase 1 gene is involved in the detoxification of methylglyoxal and methylglyoxal is directly or indirectly involved in the development of T2DM. To better understand disease etiopathology at molecular level we study single nucleotide polymorphisms. Single nucleotide polymorphisms (SNPs) are the most abundant genetic variations, they are a cause of many complicated diseases. Around 2% of the known SNPs are linked to diseases by changing amino acid and might result in non-functional protein (Alanazi *et al.*, 2011). SNPs involved in the regulation of proteins and genes can also play a role in disease development.

The data obtained for the research using computational bioinformatic tools and genetic analysis suggests that regulatory SNPs specifically intronic, 3' and 5' and missense SNPs can play important role in gene expression and protein function. They can also be consequential in prevalence and disease outbreak.

The results from computational tools predict 7 SNPs (Table 6) of importance. These SNPs are expected to alter binding sites for proteins and miRNA. Among the screened SNPs some have high regulomeDB score of 4. These seemingly less important SNPs gave hits on FuncPred and PolymiRTS. Obtained results are accordant with the hypothesis. The results from genetic analysis of missense SNP rs1038747749 through high resolution melt analysis (HRM) demonstrate more presence of risk allele in patient samples than healthy people. HRM analysis is more precise and accurate when compared to conventional PCR. Calculated p-value for this SNP is significant and represents association of SNP with disease phenotype (T2DM).

Considering the constraints, we cannot omit the fact that some seemingly non-significantly SNPs could also be associated to T2DM. Other categories of variant and SNPs can also be

analysed further. The short listed regulatory non-coding SNPs can be further studies *in-vitro* in patients with T2DM to get deeper understanding.

This study is gender non-specific, and more research can be done to check association respective to gender. Furthermore, sequencing can be performed to validate the results in more precise way. These significantly associated SNPs can be used for target therapies, personalized medicines.

Table 6: Selected associated SNPs list.

Variant ID	Alleles	Global MAF	Conseq. Type	RegulomeDB Score	SNPinfo (TFBS)	SNPinfo (miRNA)	PolymiRTS
rs16890915	A/G	0.015	3 prime UTR variant	4		Y	Y
rs75200860	T/A	0.016	3 prime UTR variant	2a			Y
rs7604	C/T	0.052	3 prime UTR variant	1f		Y	Y
rs9470916	C/A	0.129	3 prime UTR variant	4		Y	Y
rs1049346	G/A/C	0.435	5 prime UTR variant	1f	Y		
rs2277109	C/A/G	0.052	intron variant	1f	Y		
rs2490026	C/G/T	0.499	intron variant	1f	Y		

CONCLUSION AND PROSPECTIVE

Computational analysis of single nucleotide polymorphisms of Glyoxalase 1 gene predicted seven pathogenic non-coding SNPs: four 3'SNPs, one 5'SNP and two intronic SNPs. These SNPs can be potentially harmful for the regulation of gene by disturbing transcription factor binding sites or by interfering with miRNA. In the genetic association analysis of missense SNP rs1038747749; ancestral allele C is observed in healthy patients and risk allele T is observed more in T2DM patients, confirming the significant association of SNP rs1038747749 with T2DM. SNP rs753587598 showed no difference in the pattern in wild or mutant genotype in patients and controls, therefore it is not found to be associated with T2DM. Present research study can be replicated with a larger sample size. High resolution melt curve analysis followed by sequencing can help in refining of the data. Potentially damaging SNPs can also be verified by using *in-vitro* genetic association analysis.

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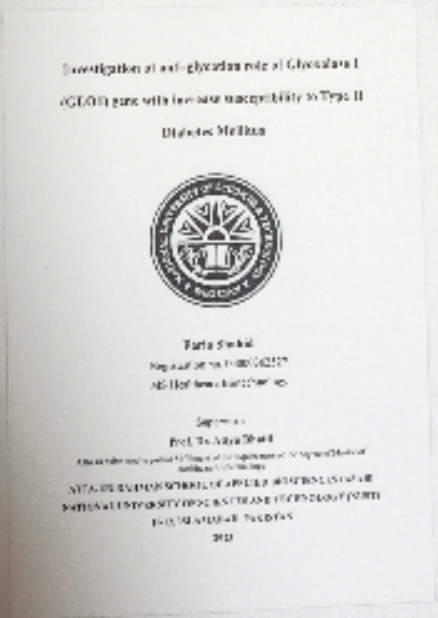

Investigation of [illegible] role of Glucokinase I
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