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Impact of genetic polymorphism of GLUT4 on response to exogenous insulin therapy in type 1 diabetic children in Kerbala province

A Thesis

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بِسْمِ اللَّهِ ا لرَّحْمٰنِ الرَّحِيم

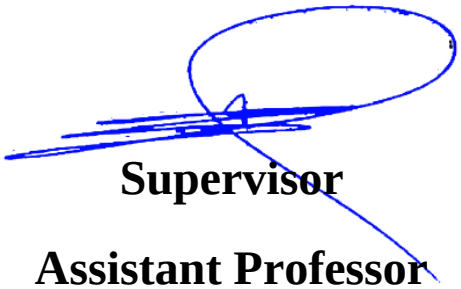
(قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَاۖ
إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ)

صدق الله العلي العظيم

سورة البقرة الاية (32)

Supervisor Certification

We certify that this thesis was prepared by (**Mohammed Suhail Abed**) under our supervision at College of Pharmacy / University of Kerbala as a partial requirement for the degree of Master of Science in Pharmacology and Toxicology.



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Dedication

To My Parents,

My Wife and

Children

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Abbreviations	**Full-Text**
AMP	Adenosine monophosphate
AMPK	5-AMP-activated protein kinase
ATP	Adenosine triphosphate
BB	Binding buffer
BMI	Body mass index
Bps	Base pairs
C3G	Exchange factor
CAP	c-Cbl-associated protein
c-Cbl	Proto-oncoprotein
cDNA	Complementary deoxyribonucleic acid
ChREBP	Carbohydrate response element-binding protein
CI	Confidence interval
CLB	Cell lysis buffer
CrkII	Adaptor protein complex
CWS	Column wash solution
CYP2D6	Cytochrome P450 family 2, subfamily D, member 6
DKA	Diabetic ketoacidosis
Dl	Deciliter
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetate

FSG	Fasting serum glucose
G6PD	Glucose-6-phosphate dehydrogenase
GLUT	Glucose transporter
HbA1c	Glycosylated hemoglobin
HLA	Human leukocyte antigen
HMIT	Protein-coupled myoinositol transporter
HWE	Hardy-Weinberg Equilibrium
IR	Insulin resistance
IRS	Insulin receptor substrate
Kg	Kilogram
Km	Michaelis constant
LSD	Least significant difference
M^2	Square meter
MAF	Minor allele frequency
MFS	Major facilitator superfamily
Mg	Milligram
mM	Millimolar
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NAT	N-acetyltransferase
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol3-kinase

PK	Proteinase K
rCGU	Regional cerebral glucose utilization
RPM	Round per minute
Rs	Reference SNP
Sirt1 deastylase	Sirtuin 1 deastylase
SAS	Statistical analysis system
SLC2A4	Solute carrier family 2, subfamily A, member 4
SNP	Single nucleotide polymorphism
T1D	Type 1 diabetes
TBE	Triesborate EDTA
TC10	Tumor cell 10
TGN	Endosomal-trans-Golgi network
TTAB	Tetra decyltrimethyl ammonium bromide
UTR	Untranslated region
UV	Ultra violet

Abstract

Background: Solute carrier family 2, subfamily A, member 4 (SLC2A4) is a gene that encodes for glucose transporter type 4 (GLUT4). Upon insulin stimulation as a result of increased blood glucose levels, SLC2A4-induced GLUT4 expression will increase. The function of GLUT4 is to facilitate glucose absorption from the bloodstream toward the cells.

Aims of Study: To evaluate the frequencies of specific selected single nucleotide polymorphisms (SNPs) (rs121434581 and rs5435) in the *SLC2A4* gene in a sample of Iraqi type 1 diabetic patients taking exogenous insulin and to evaluate the impact of these SNPs occurrence on the response to exogenous insulin therapy in those patients.

Patients and Methods: A case-control study with a total of 130 participants aged between 6 and 18 years old, both male and female, including 100 patients receiving exogenous insulin in the form of a basal-bolus treatment regimen and 30 healthy control participants. Each participant underwent biochemical testing to measure fasting serum glucose (FSG) and glycosylated hemoglobin (HbA1c) levels. To identify the SNPs rs121434581 (G > A) and rs5435 (T > C), the allele-specific polymerase chain reaction (AS-PCR) technique was employed.

Results: In patients, the genotype frequencies for rs121434581 (G > A) were 67% for homozygous wild (GG), 16% for heterozygous mutant (GA), and 17% for homozygous mutant (AA). Among healthy controls, these frequencies were 80% (GG), 10% (GA), and 10% (AA). For the rs5435 (T > C) variant, the distribution in patients was 89% for homozygous wild (TT), 6% for heterozygous mutant (TC), and 5% for homozygous mutant (CC). In the control group, the corresponding frequencies were 90% (TT), 6.6% (TC), and 3.3% (CC). Despite heterozygous genotype (GA) and homozygous genotype (AA) of SLC2A4 rs121434581 and homozygous genotype (CC) of SLC2A4 rs5435 having odds ratios higher than 1, indicating they were at

higher risk for increased FSG and HbA1c levels than wild genotypes, the values of the odd ratio were not significant.

Conclusion: Although the two SNPs of the SLC2A4 gene that were investigated in Iraqi type 1 diabetic patients affect the response to exogenous insulin, but the correlation was not significant.

Chapter One

Introduction

1.1. Type 1 Diabetes Mellitus:

Type 1 diabetes (T1D) during childhood is one of the most prevalent chronic illnesses and considered as autoimmune disease targeting pancreatic β-cells that results in lifelong absolute insulin deficiency. there were about 8.4 million individuals worldwide with type 1 diabetes: of these 1.5 million (18%) were younger than 20 years, 5.4 million (64%) were aged 20–59 years, and 1.6 million (19%) were aged 60 years or older. About two-thirds of all pediatric instances of diabetes are considered as T1D, notwithstanding the current epidemic of type 2 diabetes (Gregory *et al.*, 2022).

T1D affects 1.7 to 2.5 instances out of every 1000 people in the US, with an annual incidence of 15 to 17 cases per 100,000 people (Karvonen *et al.*, 2000). Each year, the United States sees between 10,000 and 15,000 new diagnoses of type 1 diabetes (T1D). There appear to be two childhood peaks in T1D presentation: one around age 5–7, and the other during puberty. The geographic location and seasonal variations affect the incidence of T1D. Summertime sees lower incidence rates, whereas fall and winter see greater rates. Though there are clear exceptions, some research indicates that the occurrence of this illness is positively correlated with the distance north of the equator (J. *et al.*, 1999).

The incidence of type 1 diabetes (T1D) varies by up to 400-fold across different countries that report data, and the disease's prevalence and incidence vary greatly around the globe (LaPorte, Matsushima and Chang,1995). Venezuela, China, and India have very low incidence rates of T1D (0.1 per 100,000). Sardinia and Finland have significantly higher incidence rates of the disease, close to 50 cases per 100,000 people annually. New Zealand, Canada, Great Britain, Sweden, Norway, and Portugal all had rates of over 20 cases per 100,000 people. Significant differences have been noted between adjacent regions in North America and Europe. With a

distance of less than 75 miles, the incidence of type 1 diabetes (T1D) in Estonia is less than one-third of the rate observed in Finland. While nearby Cuba has an incidence rate of fewer than 3 cases per 100,000 people, Puerto Rico has an incidence equivalent to that of the US mainland (17 cases per 100,000) (LaPorte, Matsushima and Chang, 1995). Globally, the prevalence of Type 1 diabetes is rising, with notable shifts particularly seen in young children from nations with traditionally high incidence rates (e.g., children younger than 5–7 years old in Norway). Finland has shown a 2.4% yearly increase in T1D incidence, while Norway and Sweden have recorded 3.3% annual increases in T1D rates (Karvonen *et al.*, 2000).

Merely altering one's socioeconomic situation is insufficient to account for increases in T1D incidence rates while women are disproportionately affected by many autoimmune disorders, T1D appears to affect both sexes equally. However, some findings suggest that male patients under the age of 20 have a slight excess of T1D cases (Weets *et al.*, 2001; Krischer, Cuthbertson and Greenbaum, 2004).

When considered collectively, variations in the prevalence of the disease and shifts in the incidence rates imply that the risk of T1D is influenced by a multitude of genetic and environmental factors (LaPorte, Matsushima and Chang, 1995). There is also debate on the extent of beta cell loss necessary for the development of symptoms; Recent studies suggest that 40% to 50% of beta cells are still viable at the onset of hyperglycemia. This could clarify why insulin secretion in individuals with type 1 diabetes may remain stable for prolonged periods, despite ongoing autoimmune activity. Insulin secretory function declines only when autoimmunity outpaces beta cells' capacity for regeneration (shown by a reduction in first-phase insulin production as determined by an intravenous glucose tolerance test). Following the loss of the first-phase insulin response, individuals typically experience a period of glucose intolerance, followed by a phase of clinically

"silent" diabetes. Further research focused on T1D prevention will require better knowledge of the natural course of prediabetes. Information that can guide efforts towards the objective of preventing disease includes improved disease risk markers, additional identification of environmental agents that influence the disease, and the ongoing identification of genes that might influence the disease susceptibility. Type1diabetes is regarded as a complex, multifactorial disease because it does not follow any Mendelian inheritance pattern, despite the fact that it is heavily influenced by genetic variables. The significance of environmental risk and genetic factors for type 1 diabetes was explained by several studies on familial aggregation and twins. The lifetime risk of T1D is 1 in 300 for the general population in united states, but it is 1 in 20 for Americans with a first-degree relative who has the disease (Redondo, Fain and Eisenbarth, 2001).

The concordance percentage between dizygotic twins and monozygotic twins is 6% to 10% and 30% to 50%, respectively. Eighty-five percent of T1D cases are in people who have never had the disease in their family. The parent with diabetes also affects differences in risk. Offspring of mothers with T1D are only 2% likely to get T1D, but offspring of fathers with T1D are 7% likely (Hämäläinen and Knip, 2002). The human leukocyte antigen (HLA) complex, the first locus linked to T1D susceptibility, accounts for 40% to 60% of genetic susceptibility. HLA genes are categorized into three classes, with class II genes showing the highest correlation with type 1 diabetes, for example; the influence of major histocompatibility complex allelic variability on T1D risk can be attributed to differences in the presentation of islet cell antigens, which may either promote anti-self reactivity or fail to induce regulated immune responses. This is because class II HLA genes encode for molecules that participate in antigen presentation. Thirty percent of T1D patients are heterozygous for the HLA-DR3/DR4 class II antigens, which are carried by the majority of patients (Redondo,

Fain and Eisenbarth, 2001). Three common characteristics can be seen in new-onset T1D presentations: silent diabetes, diabetic ketoacidosis (DKA), and classic new onset. DKA continues to represent 20% to 40% of all new diagnoses in several places, despite the fact that the majority of children present with typical symptoms of new-onset diabetes (Mallare *et al.*, 2003).

Less frequently diagnosed silent diabetes usually affects youngsters enrolled in diabetic research projects or adopted by households where one member has the condition already. Classic new-onset T1D in children usually manifests as weight loss, lethargy, polydipsia, polyuria, and polyphagia. Children with classic-onset T1D differ from those with DKA in that they have sufficient beta cell function preserved, preventing metabolic decompensation and the ensuing acidosis. Extended hyperglycemia is the source of characteristic diabetic symptoms. Blood glucose levels that are higher than the renal reabsorption threshold (about 180 mg/dL) cause glycosuria, which leads to osmotic diuresis, thirst, and dehydration. Weight loss is a result of several factors, including progressively decreased tissue uptake of glucose, persistent glycosuria as a result, and the breakdown of amino acids to provide gluconeogenic substrates, along with fat breakdown to supply fatty acids for ketogenesis (Dunger *et al.*, 2004).

1.2 Exogenous Insulin Therapy:

Exogenous insulin therapy involves administering insulin from external sources to manage blood glucose levels, primarily in individuals with diabetes mellitus. This approach compensates for inadequate endogenous insulin production or action. Exogenous insulin therapy is essential for individuals with type 1 diabetes, where the body's immune system destroys insulin-producing beta cells in the pancreas, necessitating external insulin for survival(Chaudhuri, Dandona and Fonseca, 2012).

There are several types of exogenous insulin, one of them is mixtard insulin 30/70 that has been used in this study; which is a biphasic insulin formulation used to manage blood glucose levels in individuals with diabetes mellitus. It combines two types of insulin to provide both immediate and extended glucose control. Mixtard insulin 30/70 consists of 30% Soluble Insulin which is a rapid-acting human insulin that begins to lower blood glucose shortly after injection, and 70% Isophane Insulin which is an intermediate-acting insulin that provides a prolonged glucose-lowering effect. This combination ensures both an immediate and sustained insulin action (Mitrushkin, 2005).

Pharmacodynamic effect of this type of exogenous insulin is characterized by facilitating glucose uptake into muscle and fat cells and inhibits hepatic glucose production, thereby lowering blood glucose levels. The biphasic nature of Mixtard 30/70 allows for onset of action which is approximately 30 minutes after subcutaneous injection, peak effect occur between 2 to 8 hours post-injection, while duration of action is up to 24 hours. From Pharmacokinetic point of view, the soluble insulin component is rapidly absorbed, reaching maximum plasma concentrations within 1.5 to 2.5 hours, while the isophane insulin component is absorbed more slowly, providing a prolonged effect. during distribution of Insulin, it exhibits minimal binding to plasma proteins, except for circulating insulin antibodies, then it will be degraded by insulin protease and into inactive metabolites. with terminal half-life ranging from 5 to 10 hours (Bock *et al.*, 2020).

1.3. Glucose Transporters:

1.3.1. Glucose as a Substrate and Regulator of Metabolic Pathways:

Glucose, which is usually found in various polymerized forms like cellulose, is a major product of carbon fixation performed by photosynthetic

organisms. As a fundamental substrate for both catabolic and anabolic processes across most living species, various mechanisms have consequently evolved to use it.

Higher organisms must adapt to external resources and engage in substantial inter-organ communication in order to maintain their energy integrity. In this situation, glucose has taken on a function as a signaling molecule that regulates energy and glucose balance. Hormone release, enzyme activity, gene transcription, and glucoregulatory neuron activity can all be regulated by glucose. The influence of glucose on the expression of lipogenic and glycolytic genes is mediated by carbohydrate response element-binding protein (ChREBP) transcription factor (Postic and Girard, 2008).

Through the glucosamine route, glucose flux controls transcription factor activity by encouraging O-GlcNAcylation (Issad and Kuo, 2008). Through the generation of NAD+, glycolysis influences Sirtuin 1 (Sirt1) deacetylase activity, a key transcriptional regulator that has come to light (Yu and Auwerx, 2009).

Histone changes brought forth by glucose metabolism through the synthesis of Acetyl-CoA lead to epigenetic regulation of gene expression (Wellen *et al.*, 2009). Increases in glucose levels can stimulate or suppress specific groups of glucose-sensitive neurons in the brain. Additionally, glucose plays a crucial role in regulating insulin release from pancreatic β-cells. These neurons are responsible for managing feeding, energy expenditure, and glucose homeostasis. The primary role of these many glucoregulatory processes is typically glucose absorption, which is controlled by the expression of glucose transporters on the cell surface in the majority of tissues (excluding hepatocytes and pancreatic β-cells). The basis for fine-tuning glucose uptake, metabolism, and signal generation to maintain cellular and overall body metabolic integrity is the availability of

numerous glucose transporter isoforms, each with unique kinetic characteristics and controlled cell surface expression (Marty, Dallaporta and Thorens, 2007).

1.3.2. Glucose Transporting Proteins:

The concept that a specific component of the cellular plasma membrane was essential for glucose transport across the lipid bilayer was initially proposed by (Lefevre, 1948). This idea aimed to clarify the isomeric specificity and saturability of glucose uptake into human red blood cells, a phenomenon observed three decades earlier.

Glucose transport kinetics across the sheep placenta were first explained by (Widdas, 1952). However, it wasn't until the 1970s that it was shown that glucose transport was facilitated by a protein within the erythrocyte plasma membrane, which could be partially purified and functionally integrated into proteoliposomes (Kasahara and Hinkle, 1977; Baldwin, Gorga and Lienhard, 1981). The red cell glucose transporter (Glut1) was cloned in 1985 (Mueckler *et al.*, 1985), and since then, 13 related members of the SLC2A (Glut) protein family have been found in humans (Uldry and Thorens, 2004).

The glut protein family is a member of the Major Facilitator Superfamily (MFS) of membrane transporters (Rédei, 2008). To date, more than 5,000 members of this family have been identified across all three kingdoms of life. No organism has been found that lacks multiple members of the MFS. Most glut proteins exhibit either symmetric or asymmetric transport kinetics and facilitate the bidirectional, energy-independent transfer of their substrates across membranes .Gluts are around 500 amino acid proteins that are expected to have one N-linked oligosaccharide and twelve transmembrane-spanning alpha helices (Joost *et al.*, 2002).

1.3.2.A. GLUT1:

Glut1 is without a doubt one of the membrane transport proteins that has been investigated the most. Over the past few decades, hundreds of investigations have been undertaken on the mechanism, structure, and kinetics of transport mediated by this protein (Carruthers *et al.*, 2009). Even with all of this focus, the exact three-dimensional structure of Glut1 or how this protein moves glucose across lipid bilayer membranes is still unknown (Mueckler and Makepeace, 2009).

Despite the fact that three bacterial components of the MFS have published crystal structures (Abramson *et al.*, 2003; Huang *et al.*, 2003; Yin, 2011), Sequence similarity absence among the several families that make up the MFS means that these data are not very useful in determining the atomic structure of glut proteins (Lemieux, 2007), therefore; the mechanism of action of glucose transporters to be understood, crystal structures with high-resolution of these proteins in various conformational states should be collected. Glut1 firstly catalyzes the rate-limiting step in the delivery of glucose, a necessary fuel for central nervous system cells. One potentially extremely helpful tactic to counteract the damage that occurs to cardio myocytes during myocardial infarction and the effects of strokes caused by arterial obstruction is the capacity to acutely upregulate Glut1 activity (Yu *et al.*, 2008).

Glut1 is often upregulated during cancer development across various tissue types. This upregulation is likely crucial for tumors to expand beyond the size constrained by their glycolytic capacity, a phenomenon known as the Warburg effect. This application is inverse and considers it evident that the rational design of isoform-specific glucose transport inhibitors is of relevance (Ganapathy, Thangaraju and Prasad, 2009).

1.3.2.B. GLUT2:

Glut2 is abundantly present in pancreatic β-cells, in the basolateral membranes of hepatocytes, and in intestinal and renal epithelial cells (Thorens, 1992). It possesses a unique high Km for glucose (about 17 mM). This guarantees quick glucose equilibration between the cell cytosol and extracellular space at all glycemic levels, whether they are physiological or related to diabetes. The rate of glucose metabolism in these cells is controlled by the glucose phosphorylation step. Therefore, in most cases, Glut2 surface expression modulation does not control metabolism until its decrease is significant enough to restrict glucose availability to hexokinases, as can occur in diabetic β-cells (Thorens *et al.*, 1990). When the luminal glucose concentration is high, Glut2 can also localize to the apical surface of the intestine, increasing the absorption of glucose (Kellett *et al.*, 2008).

Increases in blood glucose levels cause pancreatic β-cells to secrete insulin, and in hepatocytes, glucose stimulates the expression of genes related to lipogenic and glycolytic processes. The lack of Glut2 inhibits the regulation of glucose-sensitive gene expression in hepatocytes as well as the release of insulin by β-cells in response to glucose. Research with gene-knockout mice has demonstrated that Glut2 is also necessary for the operation of glucose sensors found in the central nervous system and the hepatoportal vein region. It appears that these sensors regulate insulin secretion, feeding behavior, peripheral tissue glucose absorption, and glucagon secretion (Marty, Dallaporta and Thorens, 2007).

1.3.2.C. GLUT3:

The primary neuronal glucose transporter, Glut3, is found in both dendrites and axons. Regional cerebral glucose utilization (rCGU) is correlated with the amount of Glut3 expression in various brain areas. Neurons may efficiently absorb glucose because of Glut3's high affinity for glucose (Km ~1.5 mM) and its highest estimated turnover rate among the Glut isoforms. Glut3 is extensively expressed in mouse sperm and regulates the uptake and metabolism of glucose, which is essential for maturation and motility. Glut3 is expressed in extra-embryonic tissues following implantation and in the trophectoderm during the blastocyst stage of embryonic development. Preimplantation embryos from diabetic mothers had lower levels of its expression, which increases apoptosis. Glut3 knockout in mice causes embryos to undergo apoptosis. Remarkably, compacted embryos must need a brief exposure to glucose in order to advance to the blastula stage, and this is linked to the upregulation of Glut3 expression (Brown *et al.*, 2011).

Since a glucosamine pulse also has the same effect, O-GlcNAcylation of transcription factors may play a role in initiating Glut3 expression and embryonic development. Moreover, lymphocytes, platelets, and monocytes/macrophages express Glut3. In these cells, it resides in intracellular vesicles that, upon activation, can move and fuse with the plasma membrane to ensure enhanced absorption and metabolism of glucose. It is unclear what causes Glut3 to arrive on the plasma membrane in a controlled manner. A deeper comprehension of immunological and inflammatory cell activation may result from its clarification (Simpson *et al.*, 2008).

1.3.2.D. GLUT4:

Glut4 has likely been studied more extensively than any other membrane transport protein since it was identified by James et al. as a unique glucose transporter isoform in the late 1980s (Alcover *et al.*, 2016). The considerable interest in this protein is likely due to its crucial role in maintaining glucose homeostasis in the body and its complex and elusive insulin regulatory mechanism. Insulin-induced Glut4 translocation in adipocytes and skeletal muscle from intracellular membrane compartments to the cell surface has been known since the early 1980s, when (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Wardzala and Jeanrenaud, 1981) did groundbreaking research. In the three decades that have passed, a great deal has been discovered about this regulation's specifics (Larance, Ramm and James, 2008).

The exact mechanisms by which insulin signaling components affect Glut4 trafficking, the specific cellular factors involved, and the essential structural features of Glut4 required for its unique trafficking remain unclear and highly debated. This uncertainty may be related to the lack of understanding of the cellular abnormalities underlying peripheral insulin resistance in obesity. To address this, it is crucial to determine the structure of Glut4 and how specific cellular elements interact with the transporter to control its subcellular distribution. Additionally, unraveling the complex network of signaling events triggered by insulin and other mediators that influence Glut4's interactions with regulatory elements is essential to clarify Glut4's role in insulin resistance.

The lack of understanding of the cellular abnormalities driving peripheral insulin resistance in obesity likely reflects gaps in our knowledge. To address this, it is important to determine the structure of Glut4 and explore how

specific cellular components interact with it to manage its distribution within cells. Moreover, dissecting the intricate network of signaling pathways from insulin and other mediators that influence Glut4's interactions with regulatory elements is crucial for defining its role in insulin resistance. Future research should focus on uncovering the mechanisms governing both the acute and chronic regulation of Glut4 in skeletal muscle, the main site of insulin-stimulated glucose uptake in the body (Karnieli and Armoni, 2008).

1.3.2.E. GLUT5:

Glut5 is another glucose transporter with high specificity for fructose, primarily expressed in the apical membrane of intestinal epithelial cells, where it plays a crucial role in absorbing dietary fructose. However, the physiological importance of its presence in other tissues, such as the kidney, brain, fat, testes, and muscle, remains uncertain. This is due to the low concentrations of fructose in peripheral circulation and urine (less than 0.1 mM), which are much lower than the >10 mM Km of Glut5 for fructose. Diurnal rhythm, substrate availability, and other variables control the activity of Glut5. The relationship between rising fructose consumption and an increase in the prevalence of type 2 diabetes, metabolic syndrome, and obesity has sparked interest in fructose metabolism. It's unclear, though, if fructose specifically contributes to the higher frequency of these illnesses or an overall high calorie consumption is the true cause. Nevertheless, more research on Glut5's function in extra intestinal tissues must be done by creating null mice that are unique to particular tissues (Douard and Ferraris, 2008).

1.3.2.F. GLUT9:

Long-term research has been conducted to determine the physiological substrate of the Glut9 transporter (Doblado and Moley, 2009), with the original hypothesis being that it was a transporter of glucose and/or fructose (Carayannopoulos *et al.*, 2004). However, research on human genetics unexpectedly revealed this transporter's primary role. Genome-wide association studies (GWAS) conducted by a number of organizations revealed single-nucleotide polymorphisms in the Glut9 gene associated with increased levels of plasma uric acid (Li *et al.*, 2007; Vitart *et al.*, 2008).

The fact that Glut9 was a urate transporter was thus immediately apparent. In addition to the kidney, liver, and gut, chondrocytes also contain a small amount of glut9. Glut9 inactivating mutations in human result in hypouricemia, indicating a potential involvement in renal urate reabsorption (Matsuo *et al.*, 2008). Therefore, the reason for the association between hyperuricemia and gout and single-nucleotide variations found in noncoding areas found in GWAS remains unknown. Glut9 plays a crucial role in regulating uric acid levels in mice through its expression in the liver and kidney. In the liver, where urate can access its degrading enzyme uricase, while in the kidney, it is likely involved in urate reabsorption. This regulatory function has been demonstrated by studies involving genetic inactivation of Glut9 (Preitner *et al.*, 2009).

Hyperuricemia is the result of liver-specific Glut9 gene inactivation; no other physiological problems appear to be present. As suggested by epidemiological research, this mouse model should aid in investigating the potential link between high plasma uric acid levels and atherosclerosis, hypertension, or insulin resistance (Alderman, 2002).

1.3.2.G. GLUTs 6–8 and 10–14:

The specific functions of these recently discovered Glut proteins, which were identified through human genome sequencing, are still largely unknown. Glut8 is involved in heart atrial activity and hippocampus neuronal proliferation, according to preliminary research using knockout mice (Schmidt, Joost and Schürmann, 2009), while Glut12 is involved in the control of glucose homeostasis.

One of Glut protein family is Glut7 protein, and it exhibits a lot of sequence similarities with Glut5 (Cheeseman, 2008). It expresses mainly in the large and small intestine's apical membrane and has minimal fructose and glucose transport capacity. Although Glut7's primary physiological substrate is most likely yet unknown, it is probably involved in the absorption of one or more low-molecular-weight carbon compounds from food. Proton-coupled myoinositol transporter (HMIT) Glut13 is a myoinositol transporter that is mostly expressed in the brain. It is the only glut protein that seems to have a proton-coupled symporter function (Uldry *et al.*, 2001). Although its exact function in brain myoinositol metabolism is still unknown, variations of this transporter might be associated with mood disorders due to the connection between intraneuronal myoinositol metabolism and membrane trafficking at synapses and growth cones (Hallcher and Sherman, 1980; Williams *et al.*, 2002).

Although its precise function in glucose metabolism in the testes is uncertain, Glut14, a class I protein expressed in this tissue, is likely a glucose transporter due to its high sequence similarity to Glut3 (Wu and Freeze, 2002). Although the primary physiological substrate of glut6 cDNA has not been conclusively determined, it appears to demonstrate glucose transport activity (Doege *et al.*, 2000). There are three distinct forms of Glut11, and

each one is expressed in a different type of tissue (Scheepers *et al.*, 2005). It shows measurable fructose and glucose transport activity, but once more, its primary substrate is most likely still unknown. Arterial tortuosity syndrome is caused by mutations in the Glut10 gene (Coucke *et al.*, 2006). However, the precise physiological role of Glut10 and its main substrate are still not fully understood. It is obvious that a great deal remains unknown regarding the basic functions of almost half of the members of Glut protein family (Lizák *et al.*, 2019).

1.4. Glucose Transporter Type 4 (Glut4):

1.4.1. Cellular Level of Glut4:

One important physiological factor that is constantly under strict control is the blood glucose level, one of the many homeostatic processes that the human body maintains. Since too much or too little glucose can have negative effects, it is important that the body carefully regulate this energy source. Insulin controls blood glucose levels and is influenced by the consumption of carbohydrates. Insulin controls the liver's ability to produce and absorb glucose from the bloodstream. The GLUT family of transmembrane proteins facilitates the diffusion of glucose across cell plasma membranes. Their tissue distribution and kinetics are different. Insulin-stimulated glucose transport into adipose tissue and skeletal muscle, mainly through the action of glucose transporter protein type-4 (GLUT4), is the main regulatory mechanism governing glucose uptake. A crucial element in maintaining glucose homeostasis and eliminating glucose from the bloodstream is GLUT4 (Shepherd and Kahn, 1999; Huang and Czech, 2007).

Moreover, glut4 is thought to belong to a family of 12-transmembrane glucose transporter proteins. Adipose tissue and skeletal muscle are the main places where it manifests. The specific sequences at the N- and COOH-

terminals of GLUT4 determine how responsive it is to membrane trafficking and insulin signaling. The GLUT4 mechanism of ATP-independent facilitative diffusion is responsible for glucose transit across the cell membrane. Upon entering the cell, glucose can be stored as glycogen or used for energy or lipid synthesis (Huang and Czech, 2007).

Between the intracellular domain and the plasma membrane, GLUT4 changes where it is located. It can move depending on stimulation since it is a component of an intracellular tubulo-vesicular network that is linked to the endosomal-trans-Golgi network (TGN) system.

In the absence of exercise or insulin, ninety percent of GLUT4 is found intracellularly. GLUT4 storage vesicles are exocytosed into the plasma membrane, T-tubules, and sarcolemma of skeletal muscle cells when insulin or exercise are present. Here, they can perform their glucose transport role.

An increase in the number of GLUT4 molecules on the cell surface enhances the maximum rate of glucose transport into cells. Once insulin stimulation ceases, GLUT4 is internalized into the cell as vesicles coated with clathrin form on the plasma membrane. Following internalization, GLUT4 reorganizes into intracellular vesicles and forms an attachment to early endosomes (Shepherd and Kahn, 1999; Bryant, Govers and James, 2002).

1.4.2. Glut4 Function:

Skeletal muscle cells, cardio myocytes, and adipocytes are known to contain GLUT4. Insulin-stimulated glucose absorption into muscle and fat cells is mostly caused by it. Eighty percent of glucose is absorbed by muscle cells. When skeletal muscles have a higher metabolic demand during exercise or during periods of elevated blood glucose following a

carbohydrate-rich meal, the glucose-transport system of GLUT4 can be upregulated to meet greater transport needs (Bryant, Govers and James, 2002).

1.4.2.A. Insulin-Mediated Stimulation of Glut4:

Two different signaling mechanisms can cause GLUT4 translocation, which is controlled by insulin. Lipid kinase phosphatidylinositol 3-kinase (PI3K) is one route that is involved. When insulin binds to its receptor on the surface of the target cell, the receptor undergoes a structural change, activating the tyrosine kinase domain inside the cell. Then, c-Cbl, a proto-oncoprotein, and insulin receptor substrates (IRS) are phosphorylated. IRS-1 and IRS-2 serve as the main substrates in muscle and fat cells. Effector molecules like PI3K, which have been shown to be involved in the translocation of GLUT4 to the plasma membrane, are attracted to these substrates located near the plasma membrane. The other pathway involves the proto-oncoprotein c-Cbl. Insulin stimulates a dimeric complex of c-Cbl and c-Cbl-associated protein (CAP) to migrate into lipid rafts on the cell surface. Upon phosphorylation of c-Cbl, the adaptor protein complex (CrkII) and exchange factor (C3G) of the GTPase TC10 are recruited to the lipid raft. Lipid rafts are where TC10 localizes, particularly. Consequently, C3G's activation of TC10 is an insulin-dependent mechanism that results in the translocation of GLUT4. Inhibition of this route will also reduce insulin-stimulated GLUT4 translocation in adipocytes (Bryant, Govers and James, 2002; Watson, Kanzaki and Pessin, 2004).

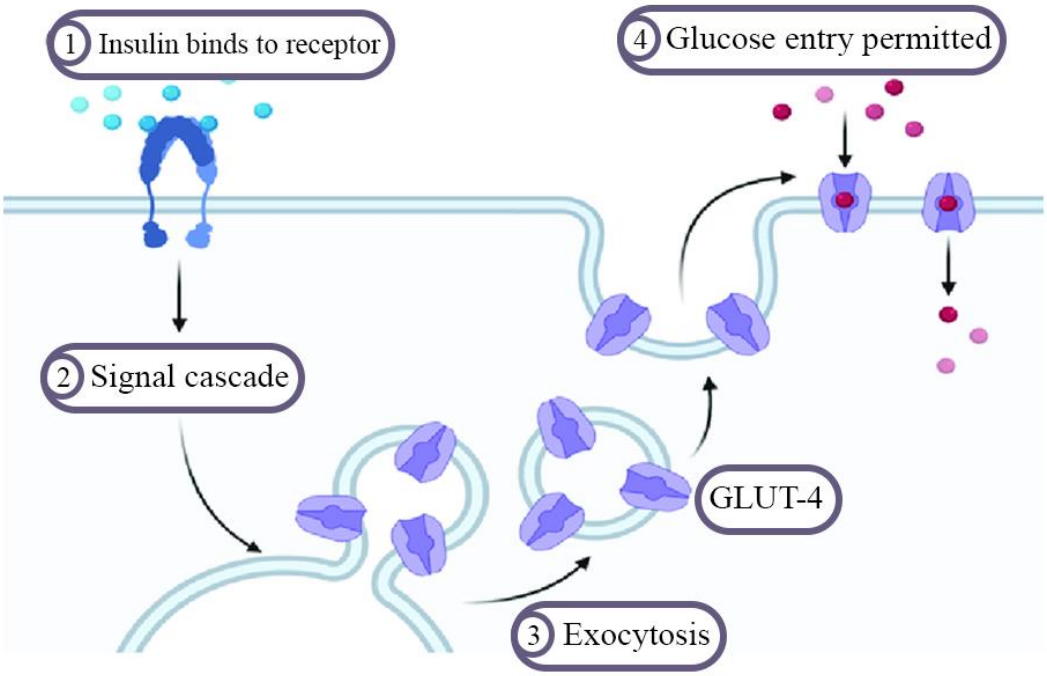

Figure (1.1): Activation of the glucose transporter GLUT4 by insulin (Singh *et al.*, 2020).

1.4.2.B. Non-Insulin Mediated Stimulation of Glut4:

In skeletal muscle, exercise promotes GLUT4 translocation to the plasma membrane. An independent mechanism from PI3K, which is required for the insulin-stimulated pathway, is responsible for this activation. In order to fulfill the increased energy requirements of skeletal muscle during activity, skeletal muscle contraction activates 5'-AMP-activated protein kinase (AMPK), which is thought to translocate exercise-responsive GLUT4-containing vesicles to the cell surface to facilitate glucose transport (Shepherd and Kahn, 1999; Bryant, Govers and James, 2002).

1.5. SLC2A4 Gene:

1.5.1. The SLC2A4 Gene definition:

The GLUT4 protein is predominantly expressed in differentiated myotubes and adipocytes. In humans, it is encoded by the solute carrier gene SLC2A4, while in mice and rats, it is encoded by the Slc2a4 gene. GLUT4, which was first identified in the early 1990s, is recognized as the glucose transporter that responds to insulin because of its unique ability to be inserted into intracellular vesicle membranes. These vesicles then translocate to the plasma membrane in response to insulin stimulation, increasing the uptake of glucose by these cells and providing the framework for postprandial glycemic control. It is stated that the insulin-induced translocation of GLUT4 is often portrayed as significantly more pronounced than it actually is in vivo. The so-called basal condition, in which insulin is absent, is compared to peak insulin action through in vitro translocation. GLUT4 translocation is extremely modest whenever the highest insulin impact is compared to basal physiological insulin concentrations (Okamoto *et al.*, 2011).

Moreover, skeletal muscle activity stimulates GLUT4 translocation, and a small amount of contractile tone is sufficient to cause significant levels of translocation. Nonetheless, a little relative translocation of GLUT4 (i.e., a small percentage associated with the overall content) might greatly enhance the absorption of glucose, preventing compromised postprandial hyperglycemia. Remarkably, a number of studies on GLUT4 translocation only investigate the total quantity of GLUT4 present in the plasma membrane. Insulin stimulates vesicle translocation; hence, despite an intact translocation mechanism, decreased insulin-stimulated plasma membrane GLUT4 content can be explained by a reduction in SLC2A4 expression (lowering GLUT4 density in the vesicles) (Machado, Shimizu and Saito, 1994).

While some research has suggested that modifications to the GLUT4 protein's molecules could affect the protein's transport kinetics, no reliable evidence has supported this theory (Marsh *et al.*, 1998; Zaarour *et al.*, 2012).

Lastly, it appears that the primary factor influencing tissue glucose elimination and, by extension, glycemic homeostasis is SLC2A4/GLUT4 expression. Therefore, research should concentrate on controlling the expression of SLC2A4/GLUT4 (Herman and Kahn, 2006).

1.5.2. SLC2A4 Expression and Insulin Resistance:

Firstly, it is crucial to recognize that insulin resistance (IR) can be characterized and examined from several perspectives without taking liver involvement into account. Initially, any disruption in the insulin signaling pathway can be used to identify insulin resistance in cells. This does not always lead to a decrease in glucose absorption; changes in SLC2A4 expression and GLUT4 translocation can maintain normal glucose transport. Second, a decrease in insulin-induced glucose uptake and SLC2A4/GLUT4 expression in muscle and/or adipose cells does not ensure a drop in plasma glucose clearance; rather, an increase in cellularity and/or tissue bulk can compensate for the deficit in cellular glucose disposal. Whole-body insulin resistance (IR), which is characterized by decreased insulin-induced plasma glucose clearance, needs to be examined in order to draw conclusions about IR and poor glycemic homeostasis. This requires analyzing the GLUT4 content of all tissues and the subsequent disposal of glucose. In non-obese individuals, skeletal muscle plays a crucial role in insulin-mediated glucose clearance; however, adipose tissue may also play a role in obese subjects (Slca and Lucia, 2013).

Interestingly, reduced GLUT4 translocation has been linked only to impaired insulin-induced glucose uptake, which is linked to defective insulin

signaling pathways. Nevertheless, the majority of research is unable to show that reduced SLC2A4/GLUT4 expression is not implicated. These studies currently focus solely on the absolute concentration of GLUT4 in the plasma membrane; they do not take into account the GLUT4 content of the entire cell or tissue. Therefore, several forms of data analysis need to be carried out in order to comprehend the role of SLC2A4/GLUT4 in insulin-mediated glucose clearance at the cellular, tissue, and whole-body levels (Seraphim, Nunes and Machado, 2001).

1.5.3. SLC2A4 Pharmacogenetic Bases:

After it was reported that genes involved in glucose metabolism were overrepresented among genes under natural selection during human evolution (Haygood *et al.*, 2007), the diversity of SLC2A4 was examined by genotyping 104 SNPs and sequencing SLC2A4 in individuals from a variety of ethnic backgrounds, including Africans, Asians, Europeans, and Latin Americans. Analysis revealed a total of 29 polymorphisms. Eight SNPs were identified in the N-terminal region, upstream of exon 7, with a minor allele frequency (MAF) greater than 5%. These included rs5415, rs5417, and rs5418 in the 5' UTR; rs222847, rs222849, and rs16956647 in intron 1; rs5435 in exon 4; and rs5436 in intron 6. Additionally, 15 SNPs had a MAF less than 5%. In contrast, only six SNPs with a MAF less than 5% were found in the C-terminal region, downstream of intron 6. The scarcity of common variations in the C-terminal region suggests natural selection may have reduced the extent of substitutions there, while the diverse pattern of frequent and rare variants in the N-terminal region aligns with neutral evolution (Tarazona-Santos *et al.*, 2010).

Although the functional ramifications of these findings are not yet known, it may be important to understand the control of *SLC2A4* gene expression (Tarazona-Santos *et al.*, 2010). Furthermore, variations in the

SLC2A4 gene's activity or expression can affect glucose metabolism and contribute to the pathogenesis of a number of metabolic diseases, including obesity, diabetes mellitus, and metabolic syndrome.

The rs5435 polymorphism (table 1.1) in the promoter region of the *SLC2A4* gene is one of the best-studied polymorphisms. This polymorphism has been linked to altered *SLC2A4* gene transcriptional activity, which affects GLUT4 expression levels. The rs5435 polymorphism has been linked in multiple studies to insulin resistance, reduced glucose tolerance, and an elevated chance of developing diabetes mellitus. For example, GLUT4 expression levels in adipose tissue were considerably lower in carriers of the T allele of rs5435, according to research by (Vionnet *et al.*, 2000), which resulted in decreased insulin-stimulated glucose absorption.

Table (1.1): *SLC2A4* gene polymorphisms, (National Center for Biotechnology Information, 2024).

Gene	**Type of SNP**	**Number of SNP**	**Total**
SLC2A4	Inframe deletion	6	3223
	Intron	1288	
	Missense	420	
	Synonymous	1509	

1.6. Rationale of Pharmacogenetic Study:

The study of hereditary variations in medication responsiveness is known as pharmacogenetics (Nebert, 1999). The word "pharmacogenomics" has been introduced due to the trend of appending the suffix "omics" to scientific fields. The latter phrase is more widely used and includes all genes in the genome that may influence drug response, whereas the former word is mostly used in regard to genes influencing drug metabolism (Evans and Relling, 1999).

In 1978, clinical DNA testing was introduced to diagnose sickle cell disease by analyzing the β-globin gene mutation (Wai Kan and Dozy, 1978). Since then, molecular testing has expanded to include population carrier-screening programs for autosomal recessive disorders (e.g., cystic fibrosis), especially among specific ethnic groups (e.g., the hemoglobinopathies in the Mediterranean), as the genetic basis for several disorders has been identified (Patrinos, Kollia and Papadakis, 2005).

In recent years, prenatal mutation studies, preimplantation genetic diagnosis, and molecular diagnosis frequently employ targeted genotyping and gene sequencing. Predictive genetic testing may be used to assess personalized disease risk, even though the majority of these testing scenarios involve Mendelian disorders. Recent genome-wide association studies (GWAS) have identified various genes and variant alleles linked to common diseases and complex traits. As the use of whole-exome and whole-genome sequencing continues to expand, it is expected that more genetic variations, both common and rare, will be discovered, significantly influencing disease phenotypes. Similarly, pharmacogenetic testing—while facing challenges related to clinical utility and acceptance—does not encounter the same level of controversy as predictive genetic testing for complex, late-onset disorders. (Relling *et al.*, 2010).

The identification of succinylcholine-induced prolonged apnea during anesthesia, caused by autosomal recessive butyrylcholinesterase deficiency. Additionally, cases of primaquine-induced hemolytic anemia, particularly among African-Americans, were later attributed to variant alleles of glucose-6-phosphate dehydrogenase (G6PD) (Beutler, 1993), and severe adverse reactions to the anti-tuberculosis drug isoniazid, which were eventually linked to variant alleles of N-acetyltransferase (NAT2) were other significant scientific discoveries in the 1950s (Blum *et al.*, 1991). Single base pair positions within genomic DNA where distinct sequence alternatives (alleles) occur in normal individuals in some population(s) and the least frequent allele has an abundance of at least 1% or higher are known as single nucleotide polymorphisms. As a result, single-base insertion and deletion variations are not regarded as SNPs. Nevertheless, insertion or deletion share many of the characteristics associated with SNPs (Brookes, 1999).

For instance, it's thought that 2–10% of people are homozygous for CYP2D6 mutant alleles that aren't functional, which makes it impossible for opioid analgesics to be effective. This would account for the wide variation in pain reduction experienced by patients taking the same dosage of codeine (Lindpaintner, 1999). Pharmacogenetics was once confined to candidate-driven research, but with the advent of next-generation sequencing technology and the mapping of the human genome, the field has evolved into pharmacogenomics. There are now more options for characterizing and classifying the degree of genetic polymorphisms in the human genome at the population level due to the abundance of genetic data (Ingelman-Sundberg, 2008).

1.7. Aims of study:

The study was designed to satisfy two specific aims:

1- To evaluate the frequencies of specific two SNPs (rs121434581 and rs5435) in *SLC2A4* gene in type 1 diabetic children taking exogenous insulin.

2- To evaluate the impact of these studied SNPs occurrence on response to exogenous insulin therapy in type 1 diabetic children.

Chapter Two

Patients, Materials, and Methods

2. Patients, Materials and Methods:

2.1. Blood Samples:

2.1.1. Patients:

This study is regarded as a case-control study and is concerned with evaluating two SNPs in type 1 diabetic patients taking exogenous insulin (Mixtard insulin 30/70) in the form of a basal-bolus treatment regimen.

The study was conducted on a group of type 1 diabetic patients (100 patients) who received exogenous insulin treatment. Fifty-six females and 44 males participated in the study, whose range of age was 6–18 years. Patients who participated in the study after their agreement were recruited from Al-Hassan Medical Center in Karbala for the period from September to December 2023. Signed informed consents from authorities in the workplaces and ethical approvals were obtained.

2.1.1.1. Patient Criteria:

2.1.1.1.A. Inclusion Criteria:

1. Diabetic patients with type 1 diabetes mellitus.
2. Patients on exogenous insulin for not less than 6 months.
3. Male and female diabetic patients.
4. Age under 18 years.

2.1.1.1.B. Exclusion Criteria:

1. Diabetic patients take an antidiabetic agent in addition to insulin.
2. Patients have been on exogenous insulin for less than 6 months.
3. Diabetic patients with type 2 diabetes mellitus.
4. Age above 18 years.

2.1.1.2. Collection of Clinical Data:

Data collected included patient's age, weight, height, duration of type 1 diabetes mellitus, duration of treatment, and any additional medications taken. This information was obtained from medical records with informed consent and directly from the patients.

2.1.1.3. Sample Collection:

A venous blood sample of approximately 2–5 ml was drawn from each type 1 diabetic patient and collected in a tube of ethylene diamine tetra acetate (EDTA) for DNA extraction and SNP detection.

2.1.1.4. Body Mass Index Determination:

The body mass index (BMI) was calculated by dividing the patient's weight in kilograms by the square of their height in meters (Masanovic, Milosevic and Bjelica, 2019), using the formula: BMI (kg/m^2) = weight (kg) / height^2 (m^2) (Wells, 2000).

2.1.2. Control Samples:

Thirty healthy people participated in the study as control after their agreement. Twelve females and 18 males participated in the study, with a range of ages (6–18).

After obtaining age, weight, and height, 2–5 ml of venous blood samples were drawn from each healthy person and collected in a tube of ethylene diamine tetra acetate (EDTA) for DNA extraction and SNP detection.

2.2. Materials:

2.2.1. Instruments:

Table 2.1 provides a detailed list of all the instruments used in this study, including their manufacturers and countries of origin.

Table 2.1: Instruments, Manufacturers, and Countries of Origin

Instrument	Manufacturer	Countries of Origin
Digital camera	Canon	England
Centrifuge	SIGMA	Germany
Hood	Lab Tech	Korea
Electrophoresis apparatus	Techin me	England
Micropipette	SLAMED	Japan
Vortex mixer	Human Twist	Germany
UV-trans illuminator	Syngene	England
Hot plate stirrer	Lab Tech	Korea
PCR- apparatus	TECHINE	England
Sensitive balance	AND	Taiwan
Distillator	GFL	Germany
Refrigerator	Hitachi	Japan
Rotisserie shaker	Waverly	United States
Cobas Integra 400+ analyzer	Roche Diagnostics	Switzerland
Water bath	Lab Tech	Korea

2.2.2. Kits and Chemicals:

Table 2.2 outlines all the chemicals and kits utilized in this study, including their manufacturers and countries of origin.

Table 2.2: Chemicals, Kits, Manufacturers, and Countries of Origin

Kits and chemicals	Manufacturer	Countries of Origin
Agarose	Intron	Korea
DNA ladder	Bioneer	Korea
Red safe nucleic acid stain	Infobio	India
Ethanol 90%	SDI	Iraq
Isopropanol	SDI	Iraq
Nuclease free water	Intron	Korea
DNA extraction kit	Promega	USA
TBE buffer	Bioneer	Korea
PCR master mix	Macrogen	Korea
Primer set tubes	Macrogen	Korea

2.3. Methods:

2.3.1. Biochemical Assay Methods:

2.3.1.1. Fasting Serum Glucose (FSG) Measurement:

Glucose level is estimated by UV, an enzymatic reference technique using hexokinase to catalyze glucose phosphorylation to glucose-6-phosphate by ATP. In the presence of NADP, glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase to gluconate-6-phosphate. without oxidizing any other carbohydrate. Photometric measurement is used to determine NADPH generation rate during the reaction, which is directly proportional to glucose concentration (Fadhil and Khalaf, 2014).

2.3.1.2. Glycosylated Hemoglobin (HbA1c) Measurement:

The blood sample collected in the EDTA tube was automatically hemolyzed on the Cobas Integra 400+ analyzer using the Cobas Integra hemolyzing reagent Gen. 2. This method employed tetradecyltrimethylammonium bromide (TTAB) as a detergent in the hemolyzing reagent, specifically to prevent interference from leukocytes, as TTAB does not lyse these cells. The assay measured all hemoglobin variants glycated at the beta-chain N-terminus, which have antibody-recognizable regions similar to HbA1c. Glycohemoglobin (HbA1c) in the sample reacted with an anti-HbA1c antibody, forming soluble antigen-antibody complexes. Due to the unique presence of the specific HbA1c antibody site on the HbA1c molecule, no complex formation occurs with the polyhapten reagent. Instead, polyhaptens react with excess anti-HbA1c antibodies to form an insoluble antibody-polyhapten complex, which is then measured using the turbidimetry method (Fadhil and Khalaf, 2014).

2.3.2. Genetic Analysis:

2.3.2.1. DNA Extraction (Meredith *et al.*, 2011)**:**

1. The sample was agitated for a minimum of 10 minutes at room temperature using a rotisserie shaker.
2. A microcentrifuge tube was prepared with 20 µL of proteinase K (PK) solution.
3. Then, 200 µL of the blood sample was added to the tube containing the PK solution and briefly mixed.
4. Next, 200 µL of cell lysis buffer (CLB) was added, and the mixture was vortexed for at least 10 seconds.
5. Blood samples were incubated at 56 °C by using a water bath.

6. After taking the tube out of the water bath, 250 µL of binding buffer (BB) was added, and the mixture was vortexed for a minimum of 10 minutes.
7. The content of the tube was transferred to the binding column, and it was centrifuged for 1 minute at maximum speed (15000 RPM) to enhance filtration (passage of lysate from the binding tube to the collection tube).
8. The collection tube containing the lysate was removed and discarded.
9. The binding column was transferred to a new collection tube.
10. 500 µL of column wash solution (CWS) was added to the binding column, which was then centrifuged at maximum speed for 3 minutes. The flowthrough was discarded.
11. Step 10 was repeated two more times, resulting in a total of three washes.
12. The binding column was placed in a clean microcentrifuge tube.
13. 75 µL of nuclease-free water was added to the binding column, followed by centrifugation at maximum speed for 1 minute.
14. The binding column was discarded, and the eluate was saved.

2.3.2.2. Allele Specific Polymerase Chain Reaction (AS-PCR)

(Wang *et al.*, 1998)**:**

2.3.2.2.A. Primers Preparation:

Polymerase Chain Reaction (PCR) was conducted using specific primers to amplify the SLC2A4 gene variants rs121434581 and rs5435. The primers were custom-designed by Al-wateen specialized laboratory in Kerbala province using Primer-BLAST software and were procured as lyophilized products from Macrogen, Korea, at various picomole concentrations. The lyophilized forward and reverse primers were

reconstituted in nuclease-free water to create a stock solution with a concentration of 100 pmol/µl (refer to Table 2.3). For the working solution, 10 µl of the stock solution for each primer was diluted with 90 µl of nuclease-free water to achieve a concentration of 10 pmol/µl. The primers were stored at -20 °C until needed. Table 2.4 provides details on the primers used for gene allele amplification.

Table (2.3): Dilution of primer set tubes.

Primer set tubes	**Dilution volume**
Rs121434581-forward G allele	250 µL
Rs121434581-forward A allele	250 µL
Rs121434581-reverse	250 µL
Rs5435-reverse T allele	300 µL
Rs5435-reverse C allele	300 µL
Rs5435-forward	300 µL

Table (2.4): Nucleotide sequence of primer set tubes.

Primer set tubes	**Nucleotide sequence**	**Product length**
Rs121434581 Forward G allele Forward A allele Reverse common	 5-CGAGTTCCAGCCATGAGCTACG-3 5-CGAGTTCCAGCCATGAGCTACA-3 5-AGGTAAGTTATGCCACTGGTGCG T-3	410
Rs5435 Reverse T allele Reverse C allele Forward common	 5-CATTTCATAGGAGGCAGCAGCT-3 5-CATTTCATAGGAGGCAGCAGCC-3 5-CCCAGGTGATTGAACAGAGCTA-3	355

2.3.2.2.B. Optimization of Polymerase Chain Reaction (PCR) Conditions:

The optimization of PCR conditions involved multiple trials to establish the optimal annealing temperature, concentrations of DNA and primers, and the number of amplification cycles (Roux, 2009). Details of the PCR components used for each amplified fragment are shown in Table 2.5, while Table 2.6 outlines the optimized PCR programs.

2.3.2.2.C. Conducting the Polymerase Chain Reaction:

The PCR was set up by combining the reaction components at the specified concentrations. Subsequently, the PCR was executed using the optimized protocols detailed in the following tables.

Table (2.5): PCR tube components and their volume.

Components	**Volume (µL)**
Forward primer	2 µL
Reverse primer	2 µL
DNA	4 µL
PCR master mix	10 µL
Nuclease free water	7 µL
Total volume	25 µL

(PCR: polymerase chain reaction. DNA: deoxyribonucleic acid).

Table (2.6): optimized PCR program.

Step	**Temperature/ °C**	**Time in seconds**	**Cycles number**
Denature template	95	300 seconds	1
Initial denaturation	95	20 seconds	30
Annealing	61.5	10 seconds	
Extension	72	15 seconds	
Final extension	72	300 seconds	1

2.3.2.3. Agarose Gel Electrophoresis (Huang, Baum and Fu, 2010):

1. To prepare the agarose gel, 1.5 grams of agarose powder were mixed with 70 milliliters of TBE (Tris-Borate EDTA) buffer (pH 8) and heated to boiling until the agarose was completely dissolved.
2. The gel solution was stirred to ensure it was well mixed and free of bubbles, resulting in a clear solution.
3. The solution was then allowed to cool to a temperature of 50–60 °C.
4. 10 μL of red-safe nucleic acid stain was added to the gel.
5. A comb was placed at one end of the tray to form wells for loading the PCR product samples.
6. The agarose solution was poured into the tray and left to solidify at room temperature for 30 minutes, after which the comb was carefully removed from the gel.
7. The gel was installed in a gel electrophoresis tank. TBE buffer was added to the tank until it rose three to five millimeters above the gel's surface.
8. Five microliters of DNA ladder were put into one agarose gel well, and five microliters of each PCR product were put into the remaining wells.
9. The voltage of the electrophoresis device was adjusted to produce an electrical field of five volts for every centimeter that separated the cathode and anode.
10. After the run was completed, a UV transilluminator set to 360 nm was used to visualize the bands.
11. A digital camera was then used to capture an image of the gel.

2.4. Statistical Analysis:

The statistical analysis of the data was performed using SAS (Statistical Analysis System, version 9.1). A one-way ANOVA, T-test and least significant differences (LSD) post hoc test were performed to assess significant differences among means (Masanovic, Milosevic and Bjelica, 2019). The odds ratio was estimated using MedCalc software. $P < 0.05$ is considered statistically significant (Bragazzi *et al.*, 2016).

Chapter Three

Results

3.1. Comparison of Biochemical and Socio-Demographic Parameters Between Control and Patient Groups:

Several parameters analyzed by a comparison of different health parameters between the control group (30 individuals) and the patients group (100 individuals). The parameters analyzed include Age, HbA1c (glycosylated hemoglobin), Fasting Serum Glucose, Height, Weight, and BMI (Body Mass Index). The mean age of the control group is 11.46 years, while the patient group has a slightly higher mean age of 12.44 years. The p-value (0.17) suggests no statistically significant difference in age between the groups. The control group has a mean HbA1c level of 4.97%, whereas the patients group has a significantly higher mean of 10.77%. The p-value (<0.0001) indicates a highly significant difference, meaning that patients have much higher HbA1c levels, which is commonly associated with diabetes. The mean fasting serum glucose level is 104.96 mg/dL in the control group and 208.28 mg/dL in the patients group. The p-value (<0.0001) confirms a significant difference. The control group has a mean BMI of 20.14, whereas the patients group has a mean of 19.41. The p-value (0.16) shows no significant difference in BMI between the groups. As shown in the following table:

Table (3.1): Comparison of Biochemical and Socio-Demographic Parameters Between Control and Patient Groups.

	Groups	N	Mean	Std. Deviation	P-value*
Age	Control	30	11.46	3.47	0.17
	Patients	100	12.44	3.38	
HbA1c	Control	30	4.97	0.41	<0.0001
	Patients	100	10.77	1.81	
FSG	Control	30	104.96	7.85	<0.0001
	Patients	100	208.28	88.30	
Height	Control	30	142.36	16.94	<0.0001
	Patients	100	143.71	17.72	
Weight	Control	30	41.70	11.72	0.96
	Patients	100	41.83	15.97	
BMI	Control	30	20.14	1.78	0.16
	Patients	100	19.41	4.03	

(HbA1c: glycosylated hemoglobin. FSG: fasting serum glucose. BMI: body mass index).

*T test was employed, with a p-value of less than 0.05 is regarded as significant.

3.2. Socio-demographic Characteristics of Study Participants:

The study included 100 patients whose ages ranged from 6 to 18 years, with an average age of 12.44 years. In comparison, the control group consisted of 30 participants aged between 6 and 18 years, with a mean age of 11.46 years. As shown in figure 3.1:

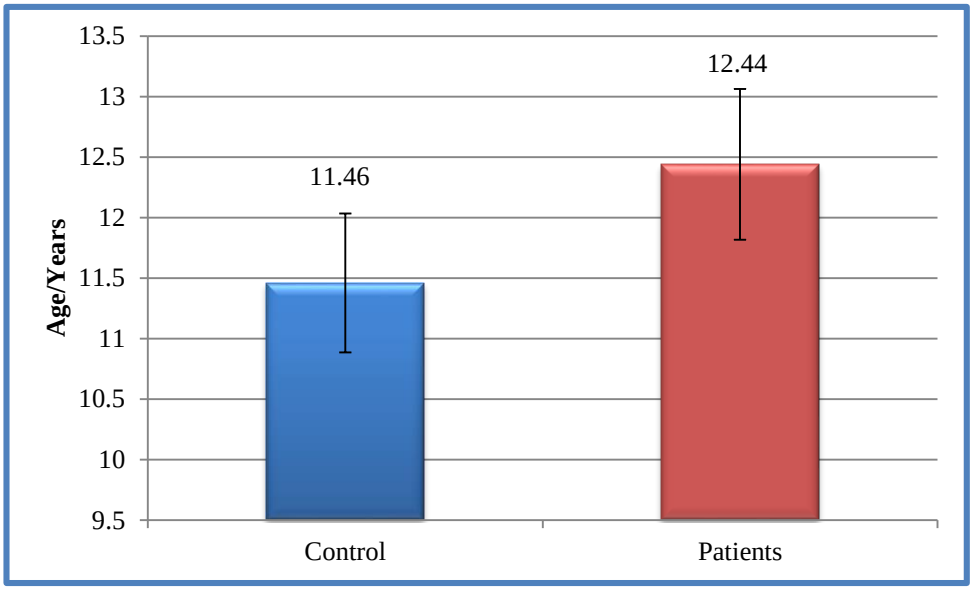

Figure (3.1): Mean of the age of the patients and control samples.

For body mass index (BMI), patients BMI ranged from 13.8 to 30 with a mean of 19.41, while the BMI of control participants ranged from 17.1 to 23.5 with a mean of 20.14. As shown in figure 3.2:

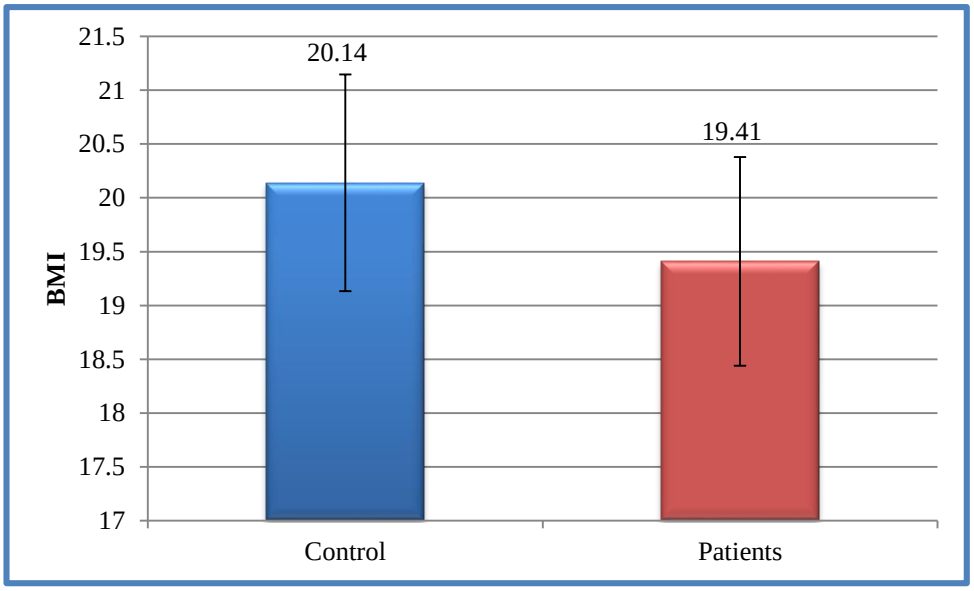

Figure (3.2): Mean of the BMI of the patients and control samples.

3.3. Biochemical Parameters:

3.3.A. Fasting Serum Glucose (FSG) of Study Participants:

Fasting serum glucose (FSG) ranged from 57 mg/dl to 400 mg/dl, with a mean of 208.28 mg/dl for the studied patients. While for the control participants, FSG ranged from 91 mg/dl to 118 mg/dl with a mean of 104.96 mg/dl. As shown in figure 3.3:

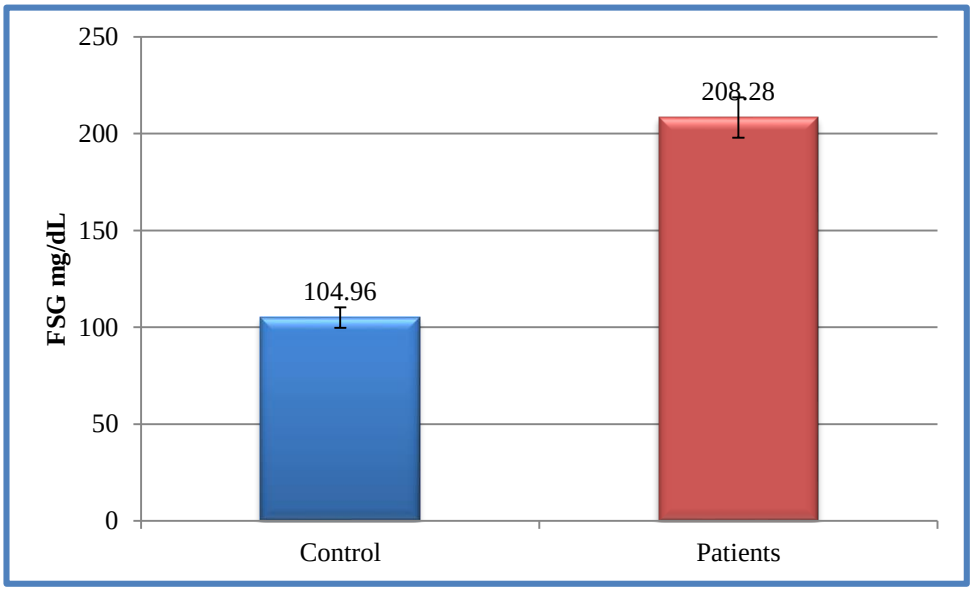

Figure (3.3): Mean of FSG of patients and control samples.

3.3.B. HbA1c of Study Participants:

HbA1c ranged from 8.5% to 15.1%, with a mean of 10.77% for patients. While for control participants, HbA1c ranged from 4.1% to 5.7% with a mean of 4.97%. As shown in figure 3.4:

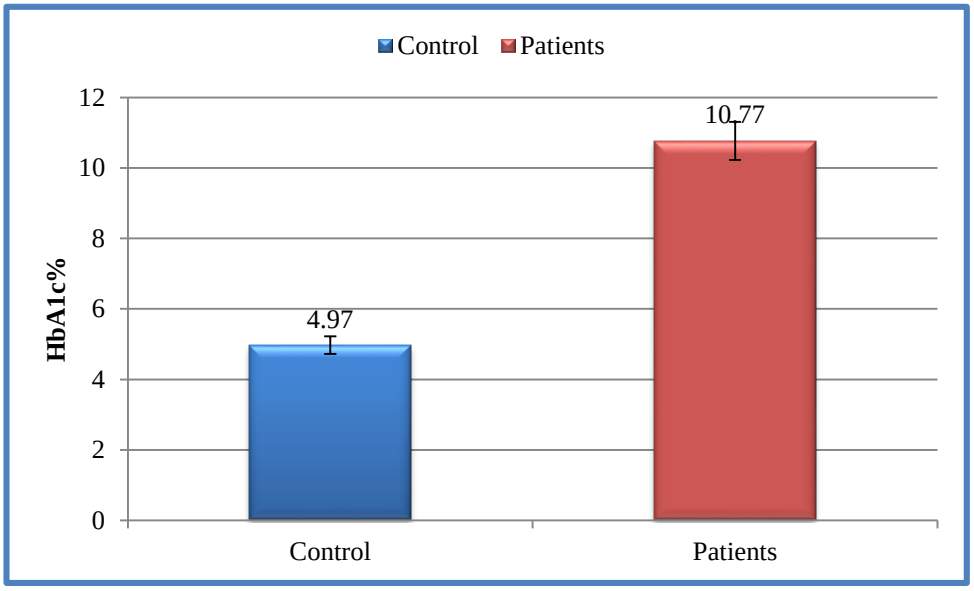

Figure (3.4): Mean of HbA1c of patients and control samples.

3.4. Least Significant Difference (LSD) among Biochemical Parameter Means for Males and Females of the Studied Participants:

HbA1c and FSG levels were compared between control and patient groups, further categorized by gender (male and female). The Least Significant Difference (LSD) values are also provided for each parameter. For HbA1c levels, the patient group shows significantly higher HbA1c levels compared to the control group because the difference in HbA1c levels for patient and control groups is more than 0.96 which is least significant difference, indicating poor glucose regulation. While for FSG levels, the patient group has significantly elevated fasting serum glucose levels compared to the control group because the difference in FSG levels for patient and control groups is more than 46.38 which is least significant difference. As explained in the following table:

Table (3.2): LSD among biochemical parameter means for males and females of studied participants.

	Groups		No.	Mean*	± Std. Deviation	± Std. Error Mean
HbA1c	Control	Male	18	4.98b	0.45	0.10
		Female	12	4.95b	0.35	0.10
	Patients	Male	44	10.83a	1.81	0.27
		Female	56	10.73a	1.83	0.24
	LSD			0.96		
FSG	Control	Male	18	104.27b	7.52	1.77
		Female	12	106.00b	8.55	2.47
	Patients	Male	44	213.90a	86.87	13.09
		Female	56	203.85a	89.94	12.01
	LSD			46.38		

(HbA1c: glycosylated hemoglobin. FSG: fasting serum glucose. LSD: Least Significant Difference)

*Means with a different letter for each parameter are significantly different ($P<0.05$).

3.5. Results of the Amplification Reaction:

3.5.1. Genotyping of rs121434581 (G>A) Genetic Polymorphism:

The gene polymorphism rs121434581 (G > A) resulted in a distinct band with a molecular size of 410 base pairs. The amplicon size was determined by comparison with a DNA ladder ranging from 100 to 1500 base pairs. As shown in figure 3.5:

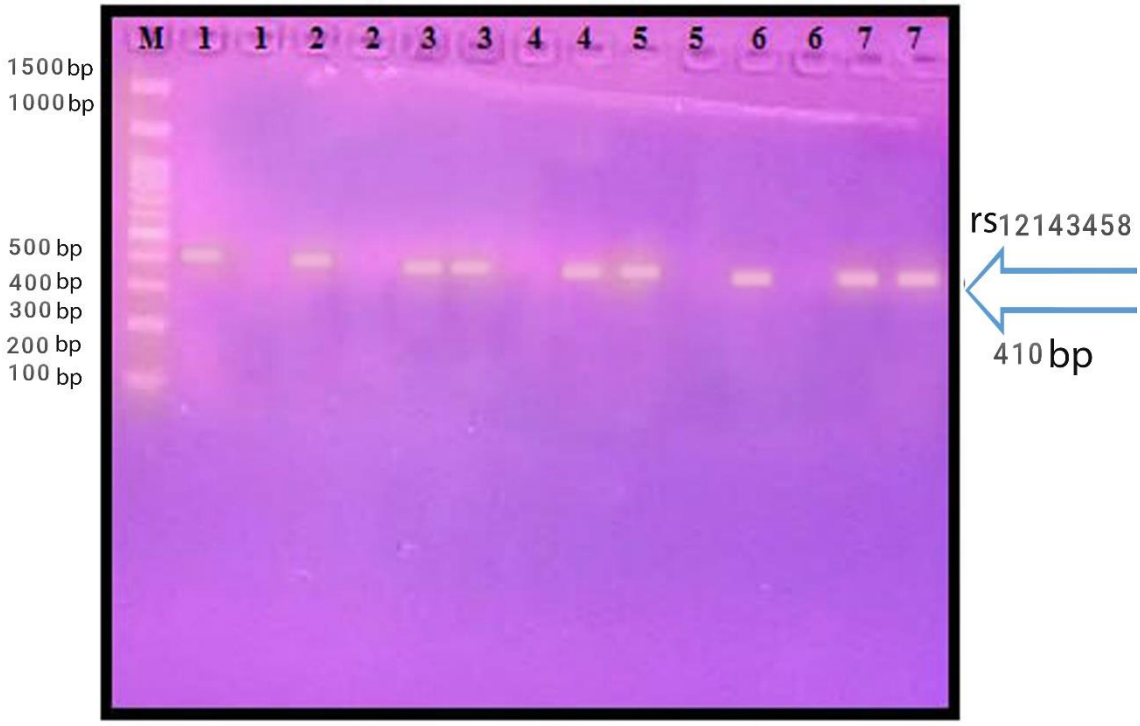

Figure (3.5): Agarose gel electrophoresis results for PCR amplification of the rs12143458 variant. DNA fragments were separated on agarose gel stained with red safe stain. Lane M represents the DNA ladder (marker) with size indications in base pairs (bp) on the left. Lanes 1–7 correspond to different samples, each showing a distinct band at approximately 410 bp, indicating successful amplification of the target region. The observed band size matches the expected PCR product for rs12143458, confirming the presence of the amplified DNA fragment.

3.5.1.A. Distribution of Allele Frequencies of rs121434581 Polymorphism (G > A) among the patients:

The patients were grouped based on three genotypes of the *SLC2A4* gene rs121434581 (G>A) polymorphism: wild type homozygous (GG), heterozygous mutant (GA), and homozygous mutant (AA). According to table 3.3, there were 67 GG genotypes (67%), 16 GA genotypes (16%), and 17 AA genotypes (17%) among 100 patients.

Table (3.3): Frequencies of alleles for the SLC2A4 rs121434581 (G > A) polymorphism among the patients.

SNP	Genotypes	Frequency	Percentage
rs121434581	GG	67	67%
	GA	16	16%
	AA	17	17%
Total		100	100%

3.5.1.B. Distribution of Allele Frequencies of rs121434581 Polymorphism (G>A) among the control participants:

The control participants were classified into three genotypes based on the *SLC2A4* gene rs121434581 (G>A) polymorphism: wild type homozygous (GG), heterozygous mutant (GA), and homozygous mutant (AA). According to table 3.4, there were 24 GG genotypes (80%), 3 GA genotypes (10%), and 3 AA genotypes (10%) among 30 control participants.

Table (3.4): Frequencies of alleles for the SLC2A4 rs121434581 (G>A) polymorphism among the control participants.

SNP	Genotypes	Frequency	Percentage
rs121434581	GG	24	80%
	GA	3	10%
	AA	3	10%
Total		30	100%

3.5.2. Genotyping of rs5435 (T > C) Genetic Polymorphism:

The rs5435 (T > C) gene polymorphism resulted in a distinct band measuring 355 base pairs. The amplicon size was determined by comparing

it to a DNA ladder ranging from 100 to 1500 base pairs. As shown in figure 3.6:

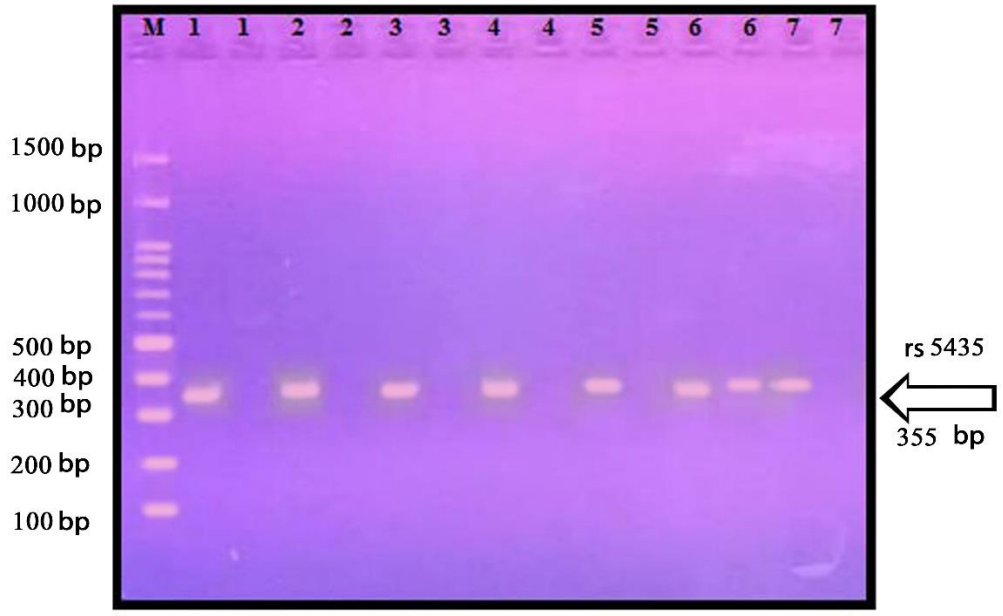

Figure (3.6): Agarose gel electrophoresis results for PCR amplification of the rs5435 variant. DNA fragments were separated on agarose gel stained with red safe stain. Lane M represents the DNA ladder (marker) with size indications in base pairs (bp) on the left. Lanes 1–7 correspond to different samples, each showing a distinct band at approximately 355 bp, indicating successful amplification of the target region. The observed band size matches the expected PCR product for rs5435, confirming the presence of the amplified DNA fragment.

3.5.2.A. Distribution of Allele Frequencies of rs5435 Polymorphism (T > C) among the patients:

The patients were classified into three genotypes for the *SLC2A4* gene rs5435 (T > C) polymorphism: wild type homozygous (TT), heterozygous (TC), and homozygous mutant (CC). According to table 3.5, there were 89 TT genotypes (89%), 6 TC genotypes (6%), and 5 CC genotypes (5%) among 100 patients.

Table (3.5): Allele frequencies of SLC2A4 rs5435 (T > C) gene polymorphism among the patients.

SNP	Genotypes	Frequency	Percentage
rs5435	TT	89	89%
	TC	6	6%
	CC	5	5%
Total		100	100%

3.5.2.B. Distribution of Allele Frequencies of rs5435 Polymorphism (T > C) among the Control Participants:

The control participants were grouped into three genotypes for the *SLC2A4* gene rs5435 (T > C) polymorphism: wild type homozygous (TT), heterozygous (TC), and homozygous mutant (CC). According to table 3.6, there were 27 TT genotypes (90%), 2 TC genotypes (6.6%), and 1 CC genotype (3.3%) among 30 control participants.

Table (3.6): Allele frequencies of SLC2A4 rs5435 (T > C) gene polymorphism among the control participants.

SNP	Genotypes	Frequency	Percentage
rs5435	TT	27	90%
	TC	2	6.6%
	CC	1	3.3%
Total		30	100%

In addition to these distribution data of the genotypes of these two SLC2A4 SNPs rs121434581 and rs5435, table 3.7 displays cross-tabulation of the genotypes of these two SNPs, showing how they distributed among the

patients group. In which GG genotype is most frequently associated with the TT genotype (58 patients), with fewer occurrence in TC (4 patients) and CC (5 patients). where us, GA genotype is mostly associated with TT (15 patients), with only 1 patient in TC and none in CC genotypes. while AA genotype is mainly linked to TT (16 patients), with only 1 patient in TC and none in CC genotypes.

Table (3.7): Cross-tabulation the genotypes of the two SLC2A4 SNPs rs121434581 and rs5435 among the patients group.

	TT	**TC**	**CC**
GG	58	4	5
GA	15	1	-
AA	16	1	-

(SLC2A4 rs121434581 genotypes: GG, GA, and AA. SLC2A4 rs5435 genotypes: TT, TC, and CC).

3.6. Least Significant Difference (LSD) among Biochemical Parameter Means for Genotypes of Studied Participants:

3.6.1. Least Significant Difference (LSD) among Biochemical Parameter Means for rs121434581 Genotypes of Studied Participants:

Levels of HbA1c and FSG in control and patient groups, furtherly categorized by different genotypes (GG, GA, AA) of the genetic variant SLC2A4 rs121434581 were analyzed by using least significant difference (LSD). For HbA1c levels, the patient group has significantly higher HbA1c levels than the control group across all genotypes because the difference in HbA1c levels for patient and control groups is more than 1.79, which is least significant difference. While for FSG levels, the patient group has

significantly higher FSG levels than the control group for all genotypes because the difference in FSG levels for patient and control groups is more than 83.19, which is least significant difference. As shown in the following table:

Table (3.8): LSD among biochemical parameter means for SLC2A4 rs124134581 genotypes of studied participants.

		rs121434581 Genotypes	**No.**	**Mean**	**± Std. Deviation**	**± Std. Error**
HbA1c	Control	GG	24	5.03b	0.40	0.08
		GA	3	5.10b	0.43	0.25
		AA	3	4.50b	0.20	0.11
	Patient	GG	67	10.72a	1.96	0.23
		GA	16	11.23a	2.08	0.52
		AA	17	10.21a	1.43	0.34
	LSD			1.79		
FSG	Control	GG	24	104.60b	7.44	1.55
		GA	3	110.66b	11.01	6.35
		AA	3	105.33b	8.02	4.63
	Patient	GG	67	210.95a	90.21	10.94
		GA	16	213.87a	92.41	23.10
		AA	17	185.64a	79.69	19.32
	LSD			83.19		

(HbA1c: glycosylated hemoglobin. FSG: fasting serum glucose. LSD: Least Significant Difference).

3.6.2. Least Significant Difference (LSD) among Biochemical Parameter Means for rs5435 Genotypes of Studied Participants:

HbA1c and FSG levels in control and patient groups, furtherly categorized by different genotypes (TT, TC, CC) of the genetic variant SLC2A4 rs5435 were analyzed by using least significant difference (LSD). For HbA1c levels, the patient group has significantly higher HbA1c levels than the control group across all genotypes because the difference in HbA1c levels for patient and control groups is more than 2.56, which is least significant difference. While for FSG levels, the patient group has significantly higher FSG levels than the control group for all genotypes because the difference in FSG levels for patient and control groups is more than 88.22, which is least significant difference. As shown in the following table:

Table (3.9): LSD among biochemical parameter means for SLC2A4 rs5435 genotypes of studied participants.

		rs5435 Genotypes	No.	Mean	± Std. Deviation	Std. Error
HbA1c	Control	TT	27	4.96b	0.43	0.08
		TC	2	5.00b	0.14	0.10
		CC	1	5.20b	0.00	0.00
	Patient	TT	89	10.81a	1.82	0.19
		TC	6	10.80a	2.12	0.86
		CC	5	10.10a	1.44	0.64
	LSD			2.56		

FSG	Control	TT	27	104.33b	7.93	1.52
		TC	2	112.50b	4.94	3.50
		CC	1	107.00b	0.00	0.00
	Patient	TT	89	209.22a	89.05	9.43
		TC	6	212.66a	82.05	33.49
		CC	5	186.20a	97.22	43.47
	LSD			88.22		

(HbA1c: glycosylated hemoglobin. FSG: fasting serum glucose. LSD: Least Significant Difference).

3.7. Hardy-Weinberg Equilibrium (HWE) for Genotypes of Studied Participants:

3.7.1. Hardy-Weinberg Equilibrium (HWE) for SLC2A4 rs121434581 Genotypes among Control Participants:

The analysis assesses whether the control group follows Hardy-Weinberg Equilibrium (HWE) for SLC2A4 rs121434581. The significant P-value (0.0009) suggests that the genotypes distribution deviates from HWE, indicating rs121434581 SNP causes significant genetic influences. As shown in the following table:

Table (3.10): HWE for SLC2A4 rs124134581 genotypes among control participants.

Genotypes	**GG**	**GA**	**AA**	**P-value**
Observed	24	3	3	
Expected	21.67	7.65	0.68	0.0009
HWE-freq.	72.25%	25.5%	2.25%	
Allele freq.	G=51 (85%)		A= 9 (15%)	

The null hypothesis is rejected because the population is not at H-W equilibrium. (HWE: Hardy-Weinberg Equilibrium).

3.7.2. Hardy-Weinberg Equilibrium (HWE) for SLC2A4 rs5435 Genotypes among Control Participants:

Assessment to determine that the control group follows Hardy-Weinberg Equilibrium (HWE) for SLC2A4 rs5435 was done. The significant P-value (0.011) suggests that the genotypes distribution deviates from HWE, indicating rs5435 SNP causes significant genetic influences. As shown in the following table:

Table (3.11): HWE for SLC2A4 rs5435 genotypes among control participants.

Genotypes	TT	TC	CC	P-value
Observed	27	2	1	
Expected	26.13	3.73	0.13	0.011
HWE-freq.	87.11%	12.44%	0.44%	
Allele freq.	T=56 (93.3%)		C=4 (6.67%)	

The null hypothesis is rejected because the population is not at H-W equilibrium. (HWE: Hardy-Weinberg Equilibrium).

3.7.3. Hardy-Weinberg Equilibrium (HWE) for SLC2A4 rs124134581 Genotypes among the Patients:

The extremely low P-value (<0.0001) indicates a major deviation from Hardy-Weinberg equilibrium. The observed genotype frequencies differ significantly from the expected ones, suggesting SLC2A4 rs121434581 causes strong genetic influences in the patients group. As shown in the following table:

Table (3.12): HWE for SLC2A4 rs121434581 genotypes among the patients.

Genotypes	GG	GA	AA	P-value
Observed	67	16	17	
Expected	56.25	37.5	6.25	<0.0001
HWE-freq.	56.25%	37.5%	6.25%	
Allele freq.	G=150 (75%)		A=50 (25%)	

The null hypothesis is rejected because the population is not at H-W equilibrium. (HWE: Hardy-Weinberg Equilibrium).

3.7.4. Hardy-Weinberg Equilibrium (HWE) for SLC2A4 rs5435 Genotypes among the Patients:

The analysis reveals a major deviation from Hardy-Weinberg Equilibrium, as indicated by the extremely low P-value (<0.0001). The observed genotype frequencies differ significantly from the expected ones, suggesting SLC2A4 rs5435 causes strong genetic influences in the patients group. As explained in the following table:

Table (3.13): HWE for SLC2A4 rs5435 genotypes among the patients.

Genotypes	TT	TC	CC	P-value
Observed	89	6	5	
Expected	84.64	14.72	0.64	<0.0001
HWE-freq.	84.64%	14.72%	0.64%	
Allele freq.	T=184 (92%)		C=16 (8%)	

The null hypothesis is rejected because the population is not at H-W equilibrium. (HWE: Hardy-Weinberg Equilibrium).

3.8. Genotype Analysis of Studied Participants by Using the Odds Ratio:

3.8.1. Analysis of SLC2A4 rs121434581 Genotypes among Studied Participants by Odds Ratio:

Genetic variations (genotypes) between the healthy control group and the patients group were compared. Specifically, three genotypes of SLC2A4 rs121434581 have been examined: wild type GG, heterozygous mutant GA, and homozygous mutant AA**,** and their distribution in both groups. By comparing to genotype GG which was regarded as reference=1, Odds ratio for genotypes GA and AA were 1.91 and 2.03 respectively, which were more than 1. As shown in the following table:

Table (3.14): Odds ratio of SLC2A4 rs121434581 genotypes among studied participants.

Genotypes	**Control**	**Patient**	**Odds Ratio (95%CI)**	**P-value**
GG	24	67	Reference=1	
GA	3	16	1.91 (0.51-7.13)	0.33
AA	3	17	2.03 (0.55-7.55)	0.29
Genotypes/Female	Control	Patient	Odds Ratio (95%CI)	P-value
GG	11	38	Reference=1	
GA	0	9	5.67 (0.31-105.13)	0.24
AA	1	9	2.60 (0.30-22.87)	0.38
Genotypes/Male	Control	Patient	Odds Ratio (95%CI)	P-value
GG	13	29	Reference=1	
GA	3	7	1.05 (0.23-4.70)	0.95
AA	2	8	1.79 (0.33-9.64)	0.49

3.8.2. Analysis of SLC2A4 rs5435 Genotypes among Studied Participants by Odds Ratio:

Genetic variations (genotypes) of SLC2A4 rs5434 between the healthy control group and the patients group were compared. Specifically, three genotypes have been examined: wild type TT, heterozygous mutant TC, and homozygous mutant CC**,** and their distribution in both groups. By comparing to genotype TT which was regarded as reference=1, Odds ratio for genotype TC was 0.91 (less than 1) and for genotype CC was 1.52 (more than 1). As shown in the following table:

Table (3.15): Odds ratio of SLC2A4 rs5435 genotypes among studied participants.

Genotypes	**Control**	**Patient**	**Odds Ratio (95%CI)**	**P-value**
TT	27	89	Reference=1	
TC	2	6	0.91 (0.17-4.77)	0.91
CC	1	5	1.52 (0.17-13.55)	0.71
Genotypes/Female	Control	Patient	Odds Ratio (95%CI)	P-value
TT	11	51	Reference=1	
TC	0	2	1.12 (0.05-24.86)	0.94
CC	1	3	0.65 (0.06-6.82)	0.71
Genotypes/male	Control	Patient	Odds Ratio (95%CI)	P-value
TT	16	38	Reference=1	
TC	2	4	0.84 (0.14-5.07)	0.85
CC	0	2	2.14 (0.10-47.13)	0.62

3.8.3. Analysis of SLC2A4 rs121434581 Genotypes among the Patients by Odds Ratio:

The distribution of SLC2A4 rs121434581 genotypes (wild type GG, heterozygous mutant GA, and homozygous mutant AA) was analyzed among males and females in the patient group. The odds ratio (OR) was calculated using the GG genotype as the reference. Compared to the GG genotype (reference = 1), the odds ratios for GA and AA genotypes were 1.02 and 1.16, respectively, both exceeding 1. As shown in the following table:

Table (3.16): Odds ratio of SLC2A4 rs121434581 genotypes among the patients.

Genotypes	Female	Male	Odds Ratio (95%CI)	P-value
GG	38	29	Reference=1	
GA	9	7	1.02 (0.34-3.06)	0.97
AA	9	8	1.16 (0.40-3.39)	0.78

While figure 3.7 displays SLC2A4 rs121434581 genotypes distribution among the patients. As shown in figure 3.7:

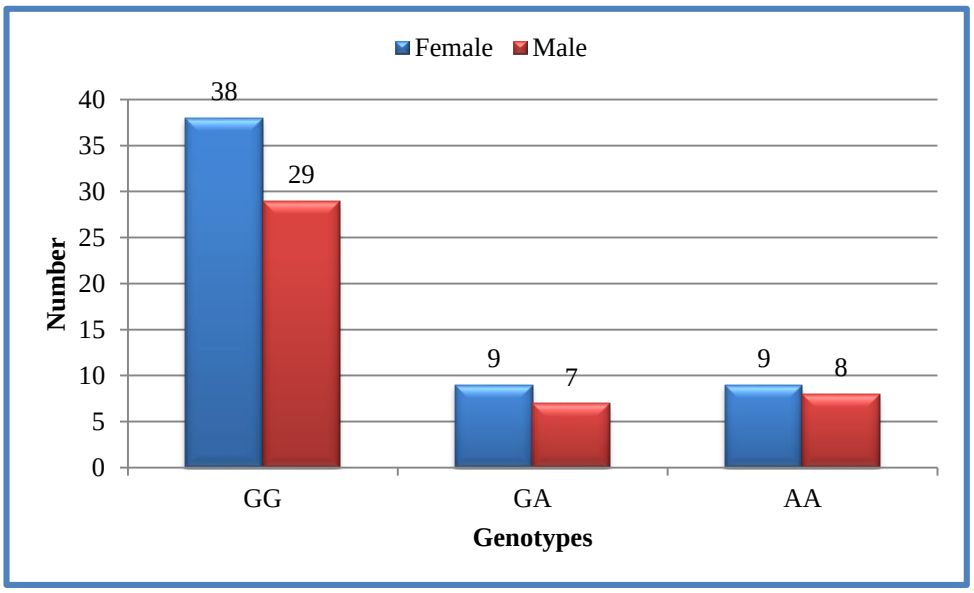

Figure (3.7): SLC2A4 rs121434581 genotypes distribution among the patients.

3.8.4. Analysis of SLC2A4 rs5435 Genotypes among the Patients by Odds Ratio:

The distribution of SLC2A4 rs5435 genotypes (wild type TT, heterozygous mutant TC, and homozygous mutant CC) among male and female patients was analyzed along with their association with the odds ratio (OR), considering the TT genotype as the reference group. Compared to the TT genotype (reference = 1), the odds ratio for the TC genotype was 2.68 (greater than 1), while for the CC genotype, it was 0.89 (less than 1). As shown in the following table:

Table (3.17): Odds ratio of SLC2A4 rs5435 genotypes among the patients.

Genotypes	Female	Male	Odds Ratio (95%CI)	P-value
TT	51	38	Reference=1	
TC	2	4	2.68 (0.47-15.43)	0.26
CC	3	2	0.89 (0.14-5.62)	0.90

While figure 3.8 displays SLC2A4 rs5435 genotypes distribution among the patients. As shown in figure 3.8:

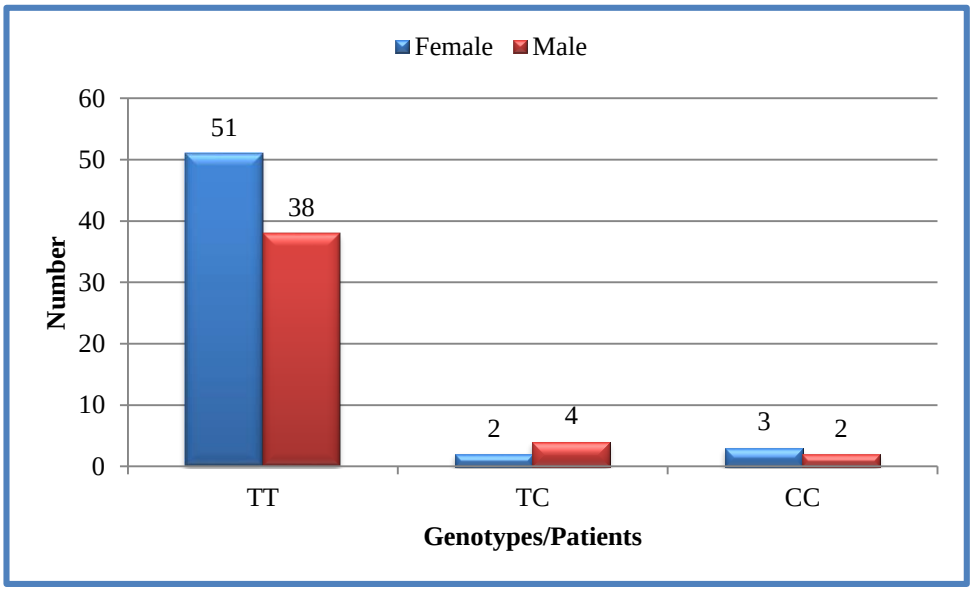

Figure (3.8): SLC2A4 rs5435 genotypes distribution among the patients.

Chapter Four

Discussion

It is generally acknowledged that insulin primarily promotes GLUT4's movement from intracellular storage vesicles to the plasma membrane. Vesicles carrying GLUT4 migrate toward the cell membrane faster in response to insulin stimulation (Olson, 2012). Without affecting the GLUT4-specific activity, more glucose enters the cells when there is more GLUT4 on the plasma membrane. GLUT4 is constantly regenerated in the plasma membrane during insulin stimulation rather than being statically preserved there (Klip, McGraw and James *et al*., 2019). The quantity of GLUT4 on the plasma membrane decreases, and the rate of movement returns to basal levels when insulin is withdrawn. The SLC2A4 gene in the human genome codes for the 509 amino acid residues that make up human GLUT4. Skeletal muscle and adipocytes are where it is primarily expressed. The sensitivity of GLUT4 to insulin signaling and membrane transport is dictated by its distinct N-terminal and COOH-terminal sequences (Watson and Pessin, 2001).

The *SLC2A4* gene, which encodes GLUT4, has many genetic polymorphisms. These variations result in reduced expression of the glucose transporter. Rs5435, which is found on exon 4 of the *SLC2A4* gene, is one of many single nucleotide polymorphisms (SNPs). The other SNP, rs121434581, is found in exon 10 of the *SLC2A4* gene. It has been agreed upon that both of these SNPs are missense SNPs (Tarazona-Santos *et al.*, 2010).

4.1. Socio-Demographic Data

The study involved 100 patients aged between 6 and 18 years, with an average age of 12.44 years. Only 2% of them were obese patients (BMI = 30), while 51% of them were underweight (BMI < 18.5).

In contrast, the ages of the 30 healthy participants who participated in the study ranged from 6 to 18 years, with a mean of 11.46 years. All of the healthy controls have a BMI less than 30, while only 4 of them were underweight (BMI < 18.5). The biochemical markers HbA1c and FSG are increased as a result of raised blood glucose levels and/or decreased SLC2A4/GLUT4 content, in which SLC2A4/GLUT4 expression appears to play a central role in tissue glucose disposal and, as a result, in maintaining glycemic homeostasis, which concur with (Karvonen *et al.*, 2000; Herman and Kahn, 2006).

According to the findings of the study presented here, there was no association between the ages of the participating patients and increased levels of biochemical markers HbA1c and FSG for those patients. P-value = 0.17, which is regarded as not significant. Besides age, the study indicates that there was no correlation between the BMI of the patients and increased FSG and HbA1c levels for those patients. P-value = 0.16, which is regarded as not significant. agreeing with (Dunger *et al.*, 2004).

4.2. Biochemical Findings:

In this study, fasting serum glucose (FSG) and glycosylated hemoglobin (HbA1c) levels for 100 patients and 30 healthy control participants were tested as biochemical parameters. FSG is routinely tested for patients as well as for control participants, as shown in Table 3.1. The FSG mean for the patients was higher than the normal range, which is for healthy control participants, in which a P-value < 0.0001 is regarded as significant.

In addition to FSG, the other biochemical parameter, glycosylated hemoglobin (HbA1c), was also tested for the patients, and the mean was

higher than the normal range, meaning the levels of FSG were higher than the normal value for the last 3 months. As explained in Table 3.1, HbA1c levels for the patients, when compared to HbA1c levels for the healthy control participants in this study, will show a significant difference in HbA1c means for control healthy participants that fall within the normal range of HbA1c. P-value < 0.0001 is regarded as significant.

On the other hand, the least significant difference among FSG means for the patients and FSG means for healthy control participants was 46.38, while the least significant difference among HbA1c means for the patients and HbA1c means for healthy control participants was 0.96. As shown in Table 3.2, the differences among FSG and HbA1c means for the patients and for healthy control participants were higher than the least significant differences. As a result of that statistical analysis, the means for each biochemical parameter are regarded as significantly different ($P < 0.05$).

4.3. Molecular Study:

4.3.1. *SLC2A4* Gene Polymorphism:

The *SLC2A4* gene rs121434581 (G > A) and the *SLC2A4* gene rs5435 (T > C) were amplified using polymerase chain reaction (PCR). For the *SLC2A4* gene rs121434581 (G > A), the results explained that the G allele was the major allele and the A allele was the minor allele in our population. While for the *SLC2A4* gene rs5435 (T > C), the T allele was the major allele and the C allele was the minor allele, which resembles the finding observed in a study involving a South Indian population found that the T allele of rs5435 was more prevalent than the C allele. The study indicated that individuals carrying the CT or TT genotypes had a higher risk of developing diabetes mellitus compared to those with the CC genotype. The odds ratio

for the CT+TT genotype was 1.26 (95% confidence interval, 1.00-1.57; P=0.043), suggesting a significant association between the T allele and increased diabetic risk (Bodhini *et al.*, 2011).

Another study conducted among Chinese diabetic patients, the T allele of rs5435 was identified as the major allele. The research demonstrated the patients with the TT genotype exhibited higher fasting blood glucose compared to those with the CC genotype. This finding suggests that the T allele may contribute to an increased risk of diabetes mellitus (Hu *et al.*, 2019).

In addition to studies identified T allele as major allele, several studied identified C allele as a major allele; such as a comprehensive study analyzing the genetic diversity of the SLC2A4 gene across various populations reported that the C allele of rs5435 was the more common allele, while the T allele was less frequent. This pattern suggests that the C allele is the major allele in these populations (Tarazona-Santos *et al.*, 2010).

For *SLC2A4* gene rs121434581, the finding of this study was agreed with initial study that has been identified the G allele as a major allele and the A allele as a miner allele (Kusari *et al.*, 1991).

4.3.2. Role of *SLC2A4* Gene Polymorphism in Increased FSG and HbA1c:

In the study presented here, the least significant differences (LSD) among biochemical parameter means for SLC2A4 rs121434581 genotypes and SLC2A4 rs5435 genotypes were calculated. For SLC2A4 rs121434581 genotypes, LSD among HbA1c means for SLC2A4 rs121434581 genotypes of the patients and healthy control participants was 1.79. while LSD among

FSG means for SLC2A4 rs121434581 genotypes of the patients and healthy control participants as detailed above was 83.19. As shown in Table 3.8, the differences among FSG and HbA1c for each SLC2A4 rs121434581 genotype for the patients and healthy control participants were higher than the least significant differences that have been calculated. As a result of that statistical analysis, the biochemical parameter means for each SLC2A4 rs121434581 genotype of study participants are regarded as significantly different ($P < 0.05$).

For SLC2A4 rs5435 genotypes, LSD among HbA1c means for SLC2A4 rs5435 genotypes of the patients and healthy control participants was 2.56. while LSD among FSG means for SLC2A4 rs5435 genotypes of the patients and healthy control participants as detailed above was 88.22. As explained in Table 3.9, the differences among FSG and HbA1c for each SLC2A4 rs5435 genotype for the patients and healthy control participants were higher than the least significant differences that have been calculated. As a result of that statistical analysis, the biochemical parameter means for each SLC2A4 rs5435 genotype of study participants are regarded as significantly different ($P < 0.05$). These findings were similar to a previous study (Salkind, 2012).

Regarding the analysis of odds ratio, as shown in Table 3.14, the SLC2A4 rs121434581 mutant genotypes for the patients (heterozygous genotype GA and homozygous genotype AA) compared to wild type GG genotype have odds ratios (with a confidence interval of 0.51–7.13 for the GA genotype and 0.5–7.55 for the AA genotype) higher than 1, indicating they were at higher risk for increased FSG and HbA1c levels than the GG genotype, suggesting patients with GA and AA genotypes are related with less GLUT4 expression and less response to exogenous insulin therapy. Farther more, homozygous mutant genotype AA has odds ratio 2.03 which

is more at risk than heterozygous mutant genotype GA, which has odds ratio 1.91. These finding suggest that the patients with AA genotype are related with less GLUT4 expression and less response to exogenous insulin therapy than the patients with heterozygous mutant genotype GA. While SLC2A4 rs121434581 GA genotypes for patient males compared to females have odds ratio near 1 (with a confidence interval 0.34-3.06) indicating they at same risk, as shown in Table 3.16. In contrast, SLC2A4 rs121434581 AA genotypes for patient males compared to females have odds ratio higher than 1 (with a confidence interval 0.40-3.39) indicating they at higher risk. these findings provide another evidence that the AA genotype is related to increased FSG and HbA1c levels and less response to exogenous insulin therapy. However, the results revealed that the odds ratio for SLC2A4 rs121434581 mutant genotypes for the patients (GA and AA) compared to wild type GG genotype is approximately twofold at risk (1.91 for GA and 2.03 for AA), but the values of the odd ratio were not significant. P = 0.33 for the GA genotype, while for the AA genotype, P = 0.29. While odds ratio for SLC2A4 rs121434581 AA genotype for patient males compared to females is at higher risk (1.16) and for GA genotype is at same risk (1.02), but the values of the odd ratio were not significant, P=0.78 for the AA genotype, while for the GA genotype, P=0.97. The findings of this study were agreed with (Kalra, 2016).

For SLC2A4 rs5435 mutant genotypes for the patients compared to wild type TT genotype, as shown in table 3.15; heterozygous genotype TC has an odd ratio (0.91 with a confidence interval of 0.17–4.77) lower than 1, indicating it is at lower risk for increased FSG and HbA1c levels. These findings suggest patients with heterozygous genotype TC are related to more expression of GLUT4 and more response to exogenous insulin therapy. However, the results revealed that the odd ratio for SLC2A4 rs5435 mutant

heterozygous genotype TC is at lower risk, but the value of the odd ratio was not significant. P=0.91. Whereas homozygous genotype CC has an odd ratio (1.52 with a confidence interval of 0.17–13.55) higher than 1, indicating it is at higher risk for increased FSG and HbA1c levels than the TT genotype. The findings of the study presented here explain that the patients with homozygous genotype CC are related to less GLUT4 expression and less response to exogenous insulin therapy compared to TT genotype. However, the results revealed that the odd ratio for SLC2A4 rs5435 mutant homozygous genotype CC is at risk, but the value of the odd ratio was not significant. P=0.71. While for SLC2A4 rs5435 TC genotype patient males compared to females, as shown in table 3.17; heterozygous genotype TC has an odd ratio (2.68 with a confidence interval of 0.47–15.43) higher than 1, indicating it is at higher risk for increased FSG and HbA1c levels, but the value of the odd ratio was not significant. P=0.26. Whereas homozygous genotype CC has an odd ratio (0.89 with a confidence interval of 0.14–5.62) lower than 1, indicating it is at lower risk for increased FSG and HbA1c levels than the TT genotype. However, the results revealed that the odd ratio for SLC2A4 rs5435 homozygous genotype CC is at lower risk, but the value of the odd ratio was not significant (P=0.9). Odds ratio statistical test that had been employed in this study was concurrent with (Kalra, 2016).

4.4. Conclusion:

1. *SLC2A4* gene polymorphism was detected with variable frequencies and different genotypes in diabetic children in Kerbala province. The allele frequency of SLC2A4 rs121434581 was higher than the allele frequency of SLC2A4 rs5435.
2. Homozygous mutant type (AA) of *SLC2A4* gene polymorphism rs121434581 was more prevalent than heterozygous mutant type (GA) of the same SNP.
3. Although the two SNPs of the *SLC2A4* gene that were investigated in diabetic children affect the response to exogenous insulin, but the correlation was not significant.

4.5. Recommendations:

1. Additional studies including a larger diverse sample size for more robust findings.
2. Investigation including the genetic influences of other types of SNPs regarding the *SLC2A4* gene to furtherly illustrate the impact of the SNPs on the insulin therapy response in type 1 diabetic children.
3. Focus on other specific genetic polymorphisms related to other type of glucose transporters involved in insulin response (e.g., GLUT2).

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Appendices

جمهورية العراق

محافظة كربلاء المقدسة
دائرة صحة كربلاء المقدسة
مركز التدريب والتنمية البشرية
شعبة ادارة البحوث والمعرفة

Holy Karbala Governorate
Karbala Health Directorate
Training and Human Development Center
Research and Knowledge Management Division

العدد: ٣٠٦١
التاريخ / ١٠ / ١٧ / ٢٠٢٣

دائرة صحة كربلاء المقدسة
قسم التدريب والتنمية البشرية

إلى / جامعة كربلاء/ كلية الصيدلة
الموضوع /تسهيل مهمة

تحية طيبة....

كتابكم المرقم (د.ع/١٦٥٣/٦ في ٢٠٢٣/٩/١٣)

نود إعلامكم بأنه لا مانع لدينا من تسهيل مهمة طالب الدراسات العليا / ماجستير (محمد سهيل عبد مطلك) لإنجاز بحثه:

"Impact of genetic Polymorphism of GLUT4 on response to exogenous insulin therapy in type 1 diabetic Iraqi patients"

في مؤسستنا الصحية/ مدينة الامام الحسين(ع) الطبية وبأشراف الدكتور (محمد فراس العبادي)على ان لا تتحمل دائرتنا اي نفقات مادية مع الاحترام .

الدكتور
نعيم عبيد الشهداني
طبيب اختصاص

الدكتورة
تقوى خضر عبد الكريم
مدير مركز التدريب والتنمية البشرية
٢٠٢٣/ ١٠/ ١٧

نسخة منه الى
مدينة الامام الحسين (ع) الطبية/ اجراء اللازم مع الاحترام.
مركز التدريب والتنمية البشرية/ شعبة ادارة البحوث والمعرفة مع الاوليات

Ministry of Health & Environment
Karbala Health Directorate
Training and Human Development Center
Research Committee

Form number 53

Decision number:2023195

Date 17/10/2023

Research committee decision

The Research Committee of Karbala Health Directorate has examined the research protocol number(2023195Karbala) entitled:

"Impact of genetic Polymorphism of GLUT4 on response to exogenous insulin therapy in type 1 diabetic Iraqi patients"

Submitted by researchers: **Mohammed Suhail Abd** to the research and Knowledge Management Unit at the Training and Human Development Center of Karbala Health Directorate on 17/10/2023

The unit has decided to:

*** Accept the above-mentioned research protocol as it meets the standards adopted by the Ministry of Health for the implementation of research, and there is no objection to implementing it in the Directorate's institutions.**

الدكتور
نعيم عبيد الشهداني

Rapporteur of the committee

17/10/2023

Notes:

- The committee member (Dr. Taqwa Khudhur Abdulkareem)/ committee rapporteur (Dr. Naeem Obaid. Talal) were authorized to sign this decision on behalf of the remaining members of the committee under the rules of procedures of the research committee.
- The research committee approval means that the research project submitted to the aforementioned committee has fulfilled the ethical and methodological standards adopted by the Ministry of Health for conducting a research. As for the implementation of the research, it depends on the researchers adherence to the instructions of the health institution in which the research will be implemented as well as the laws, instructions and recommendations in force that govern the practice of medical and health action in Iraq.

الخلاصة

المقدمة: SLC2A4 هو جين يشفر لناقل الجلوكوز النوع 4 (GLUT4). يُعبر عن هذا النوع من الناقل بشكل رئيسي في خلايا العضلات الهيكلية والخلايا الدهنية وخلايا القلب العضلية. عند تحفيز الأنسولين نتيجة زيادة مستويات الجلوكوز في الدم، يزداد التعبير عن GLUT4 الناتج عن SLC2A4. وظيفة GLUT4 هي تسهيل امتصاص الجلوكوز من مجرى الدم نحو الخلايا. قد تتسبب تعدد الأشكال في جين SLC2A4 في انخفاض التعبير عنGLUT4 ، مما يؤدي إلى ارتفاع مستويات الجلوكوز في الدم. نتيجة لذلك، يرتفع مستوى الهيموغلوبين السكري (HbA1c) وينخفض تأثير علاج الأنسولين الخارجي الذي يتناوله الاطفال المصابين بالسكري من النوع الأول في كربلاء.

هدف الدراسة: تقييم تواتر نوعين محددين من تعدد الأشكال الجيني (rs12143458 و rs5435) في جين SLC2A4 لدى الاطفال المصابين بالسكري من النوع الأول في كربلاء الذين يتناولون الأنسولين الخارجي، وتقييم تأثير حدوث هذين النوعين من تعدد الأشكال الجيني على استجابة هؤلاء المرضى لعلاج الأنسولين الخارجي.

المرضى والأساليب: دراسة حالات مرضية ومجموعة سيطرة شملت 130 مشاركًا تتراوح أعمارهم بين 6 إلى 18 عامًا، ذكورًا وإناثًا، بما في ذلك 100 مريض يتلقون الأنسولين الخارجي في شكل نظام علاج بالأنسولين الأساسي والمكمل و30 مشاركًا من الأصحاء كمجموعة سيطرة. تم إجراء اختبارات كيميائية حياتية لكل مشارك لتحديد مستويات الجلوكوز في المصل عند الصيام (FSG) والهيموغلوبين السكري(HbA1c). تم استخدام تقنية تفاعل البوليميراز المتسلسل الخاص بالأليل (AS-PCR) للكشف عن تعدد الأشكال الجيني rs121434581 G > A و rs5435 T > C.

النتائج: كان توزيع الأنماط الجينية لموضع rs121434581 G > A للمرضى هي 67 (67%)، 16 (16%)، و 17 (17%) للنوع السائد النقي (GG) ، والنوع الطافر الهجين (GA) ، والنوع الطافر النقي (AA) على التوالي. أما بالنسبة للمشاركين الأصحاء، فكانت التوزيعات 24 (80%)، 3 (10%)، و 3 (10%) للنوع السائد النقي (GG) ، والنوع الطافر الهجين (GA) ، والنوع الطافر النقي (AA) على التوالي. وكان توزيع الأنماط الجينية لموضع rs5435 T > C للمرضى هي 89 (89%)، 6 (6%)، و 5 (5%) للنوع السائد النقي (TT) ، والنوع الطافر الهجين (TC) ، والنوع الطافر النقي (CC) على التوالي. أما بالنسبة للمشاركين الأصحاء، فكانت التوزيعات 27 (90%)، 2 (6.6%)، و 1 (3.3%) للنوع السائد النقي (TT) ، والنوع الطافر الهجين (TC) ، والنوع الطافر النقي (CC) على التوالي. على الرغم من أن النمط الطافر الهجين GA والنمط الطافر النقي AA لموضع SLC2A4 rs121434581 والنمط الطافر النقي CC لموضع SLC2A4

rs5435كان لديهم نسب أرجحية أعلى من 1، مما يشير إلى أنهم كانوا في خطر أكبر لزيادة مستويات الجلوكوز في البلازما (FSG) ومستويات الهيموجلوبين السكري (HbA1c) مقارنة بالأنماط السائدة، إلا أن قيم نسب الأرجحية لم تكن ذات دلالة إحصائية.

الاستنتاج: ان التعددين الاثنين في الاشكال الجينية في جين SLC2A4 اللذين تم اكتشافهما في الاطفال المصابين بالسكري من النوع الأول في كربلاء لم يكن لهما تأثير كبير على الاستجابة للأنسولين الخارجي.

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تأثير تعدد الاشكال الجيني لـ GLUT4 على الاستجابة للعلاج بالأنسولين الخارجي لدى الأطفال المصابين بالسكري من النوع الأول في محافظة كربلاء

رسالة

مقدمة الى مجلس كلية الصيدلة / جامعة كربلاء

كجزء من متطلبات نيل درجة الماجستير في علم الادوية والسموم

بواسطة

محمد سهيل عبد

بكالوريوس صيدلة (جامعة كربلاء - 2012)

دبلوم عالي ادوية و سموم (جامعة بغداد -2018)

إشراف

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