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The Influence of Polymorphisms in the SLC5A2 Gene on Response to Empagliflozin Treatment in Patients with Diabetes Type 2

A Thesis

Submitted to the Council of College of Pharmacy/ University of Karbala as Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmacology and Toxicology

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

{ قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ الْعَلِيمُ الْحَكِيمُ }

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Dedication

To my beloved family, who have been my source of inspiration and gave me unconditional love, strength, and support every time.

To my friends, and colleagues who shared their advice and encouragement with me to go on.

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Abbreviations

Abbreviations	Meaning
4-AAP	4-aminoantipyrine
A	absorbance
AA	mutant allele
ADA	American Diabetes Association
ATP	adenosine triphosphate
B	blank
BMI	Body Mass Index
BMP	basic metabolic panel
BUN	blood urea nitrogen
CE	cholesterol esterase
CHOD	cholesterol oxidase
CMP	comprehensive metabolic panel
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
eAG	Estimated average glucose
EDTA	Ethylenediamine tetraacetic acid
eGFR	estimated glomerular filtration rate
EMA	European Medicines Agency
FBG	Fasting Blood glucose
FDA	Food and Drug Administration
FSI	Fasting Serum Insulin
GA	heterozygous allele
GDM	Gestational diabetes mellitus
GG	wild allele
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
HbA1c	Glycated haemoglobin
HF	heart failure
IDF	the International Diabetes Federation

KRW	Korean Republic won
MENA	Middle Eastern and North Africa
Met	amino acid methionine
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate hydrogen
NS	Non-Significant
OR	Odd Ratio
PCR	Polymerase Chain Reaction
POD	peroxidase enzyme
R	Correlation coefficient
rs	reference SNP
S	Significant
SD	Standard Deviation
SGLT2	Sodium dependent glucose co-transporter 2
SL5A2	soluble carrier family 5 member 2
SNP	Single Nucleotide Polymorphisms
SPSS	Statistical Package for the Social Sciences
T	test
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TC	total cholesterol
TG	Triglyceride
Try	amino acid tryptophan
TTAB	tetradecyltrimethylammonium bromide
UV	ultraviolet
Val	amino acid Valline
WHO	World Health Organization

Abstract

Background: Diabetes mellitus (DM) is a condition of high blood sugar that occurs due to insulin resistance syndrome and its deficiency due to genetic or environmental influences. Diabetes is classified into four types, and type 2 diabetes is the most common in the world. Type 2 diabetes results from a combination of two primary factors: defective insulin secretion by pancreatic beta cells and the inability of insulin-sensitive tissues to respond appropriately to insulin.

Empagliflozin is a relatively new medication that improves glycemic management by increasing urine glucose excretion and inhibiting the sodium-glucose cotransporter 2 (SGLT2).

Aims of study: - To study how the genetic variation of the sodium–glucose cotransporter 2 effects on response of empagliflozin treatment in type 2diabetes.To investigation the relationship between single nucleotide polymorphisms (SNPs) in the soluble carrier family 5 member 2 SL5A2 gene encoding SGLT2 (reference SNP 121918621) and (reference SNP 138803748) with effect of empagliflozin in patients with type 2 diabetes 2.

Method; Cross sectional study with a total of 160 participants, both male and female, included 50 healthy individuals the age range of 30 to 50 years (control group) and 110 diabetic patients type 2 ranging in age from 29 to 73years who were selected during their outpatient clinic visits and every patient received a daily dose of 10 mg of empagliflozin as monotherapy. Biochemical tests were performed on each participant to determine glycemic assessment, kidney function test, lipid

function test and a genetic study for the SLCSA2 (rs 121918621) & (rs 138803748) polymorphism

Result: The results of the study revealed that there were statistically significant differences in blood sugar indicators, body mass, and kidney indicators between the two study groups, while in lipid profile, in which no significant difference was observed between the two study groups. The blood sugar, creatinine and urea indices among diabetic patients were significantly higher than the healthy groups. In this study, genetic analysis of both alleles (rs121918621 and rs138803748) showed that wild type alleles (GG) are more prevalent than heterozygotes(AG) and mutant (AA) types. The genetic distribution of SLC5A2 (rs121918621) and (rs138803748) showed significant differences in the level (Serum creatinine, Blood urea) in both SNPs, while there was no significant difference in the level of (Fasting blood glucose, Serum Sodium, TG) in both SNPs

Conclusion: The SLC5A2 polymorphisms (rs121918621) & (rs138803748) can be regarded as one of the genetic variables causing the variation response in Iraqi diabetic patients on empagliflozin treatment.

Chapter One

Introduction

1. Introduction

1.1 Diabetes mellitus

Diabetes mellitus, is a type of resistance to insulin condition which results in elevated blood sugar levels over time. Diabetes cannot be spread to others. We can refer to it as insulin-dependent disorders (Uddin, Afroz et al. 2022) . The global diabetes prevalence in 2019 is established to be 9.3% (463 million) rising to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045 (Saeedi, Petersohn et al. 2019). Among Korean individuals aged 30 years or older, the incidence of diabetes mellitus was 14.5% in 2019 (Jeong and Kang 2022). Data from national health insurance shows that the cost of treating diabetes mellitus in 2019 was KRW 2.7 trillion, which is a 17-fold increase from KRW 160 billion in 2002(Jeong and Kang 2022).

Diabetes mellitus (DM) is a chronic condition that is common and poses serious risks to an individual's life. It is one of the main causes of death globally and a major contributor to heart attacks, stroke, blindness, renal failure, and lower-limb amputation (Mannino, Andreozzi et al. 2019).

Certain symptoms, such as increased hungry, frequent urination, weight loss, and blurred visions, are suspected of diabetes mellitus. It is linked to a lower life expectancy, serious health problems caused by particular microvascular function complications, a higher risk of macrovascular problems like peripheral vascular disease, ischemic heart disease, and stroke, and a lower quality of life overall (Rani 2021).

Diabetes mellitus (DM) is a hyperglycemic condition or metabolic disorder resulting from a combination of insulin resistance syndrome and a deficiency in

insulin, which may be attributed to genetic or environmental factors(Ramasubbu and Devi Rajeswari 2022).

Elevated blood sugar levels, known as hyperglycemia, represent a predominant characteristic shared by both type 1 and type 2 diabetes mellitus. This sustained hyperglycemia can give rise to severe complications, as it exerts a gradual and chronic influence on the human body (Abbas and El-Yassin 2022). Cell necrosis or apoptosis can occur due to prolonged hyperglycemia, resulting in oxidative damage at the levels of DNA, lipids, and proteins(Babel and Dandekar 2021). Moreover, hyperglycemia disrupts the insulin signal transduction pathway, thereby reducing glucose synthesis in the liver while simultaneously increasing glucose uptake by fat or muscle cells(Liu, Yu *et al*. 2021). Early detection and efficient management of hyperglycemia, including appropriate medication treatment, are essential for prevention due to the cellular pathological damage and the development of microvascular and macrovascular complications (Alexandru, Procopciuc *et al*. 2022).

Furthermore, the diabetes mellitus epidemic is on the rise in developing countries, with 2.2 million deaths attributed to inadequate diabetic control. Diabetes is a significant contributor to increased mortality and morbidity in these regions (Paleeratana 2019).

Lin *et al*. (2020) founded that diabetic patient have almost twice the mortality as compared with their healthy counterparts. Diabetes control and good diabetes health are necessary for quality of life and disease maintenance (Karimy, Koohestani *et al.* 2018).Adherence to behavior self-care can decrease from complications of diabetes by up to 50%.The critical aspect of controlling the disease is self-management and guaranty favorable health result. Also , according to

previous studies that show uncontrolled diabetes is a significant problem and could lead a lot of health problems as nephropathy, retinopathy, and cardiovascular diseases and can be costly (Karimy, Koohestani et al. 2018, Mikhael, Hassali et al. 2020).

1.1.1 Types of Diabetes Mellitus

DM is characterized by complex pathogenesis and wide range of presentation and classification of this disorder is arbitrary but nevertheless helpful, and it is frequently impact by the physiological conditions present at the time of diagnosis and evaluation. The classification is useful in the clinical assessment of disease and based on both the pathogenesis of disease, the etiology and for determining the good therapy. According to that, diabetes can be classify into four main categories or types: type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), diabetes caused or associated with certain specific conditions, pathologies, and/or disorders and finally gestational diabetes mellitus (GDM) (Gojnic, Todorovic et al. 2022).

1.1.1.1 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) is a prevalent autoimmune chronic disease that often affects young individuals. It is characterized by the destruction of pancreatic endocrine β-cells responsible for insulin production, particularly in specific regions of the pancreas known as the islets of Langerhans(Roep, Thomaidou et al. 2021). As a consequence, the body undergoes hyperglycemia and experiences insulin deficiency. The life-threatening complications of diabetes necessitate the ongoing care of type 1 diabetes mellitus (T1DM) patients, primarily through insulin injections. This life-saving exogenous insulin replacement poses a chronic and costly burden for individuals with diabetes. Seeking alternative therapeutic options

has become a focal point in this field. Advances in molecular biology technologies and microfabrication have opened up promising avenues. Islet transplantation, for instance, has emerged as an effective treatment to restore normal blood glucose regulation in T1DM patients(Paez-Mayorga, Lukin *et al*. 2022). Nevertheless, the potential of this technique is hindered by challenges such as extensive islet apoptosis, limited islet availability, and poor islet vascular engraftment. Addressing these unresolved issues is imperative before a potential cure for T1DM can become a reality (Rodrigues Oliveira, Rebocho et al. 2023).

1.1.1.2 Type 2 Diabetes Mellitus

A chronic increase in blood glucose levels or diabetes gradually occur as result of insulin deficiency secondary to a progressive decline of ß-cell function or insulin resistance (or both) (Pérez-Pevida, Escalada *et al*. 2019). Type 2 diabetes mellitus (T2DM) is the major type of diabetes around the world. It is caused by the body's ineffective use of insulin added to a slow progressive loss of pancreatic β-cells(Sivakumar, Prabhawathi et al. 2021).

This kind of diabetes was previously mostly seen in adults. Nevertheless, current information from the World Health Organization (WHO), there is an increasing manifestation of this condition in children. Over the past 30 years, the world has witnessed a continuous rise in the prevalence of diabetes, especially in low- and middle-income countries, representing the most rapid growth in this context (Harding, Weber et al. 2024).

In the Middle Eastern and North Africa (MENA) region, the prevalence of diabetes was recorded at 12.2%, and it is anticipated to escalate significantly, reaching 96.2% by the year 2035(Lester 2014). An increase in blood glucose levels

can result in diabetes-related complications if not properly managed (Kumar, Saha *et al*. 2020). By utilizing HbA1c, the glycemic control of patients with type 2 diabetes mellitus (T2DM) can be assessed, with the target set at less than 7%. A high HbA1c level exceeding 7% puts individuals with T2DM at risk of developing various complications associated with diabetes. These complications may include vision loss, heart attacks, strokes, leg amputations, kidney failure, and potential nerve damage (Jefferies, Rhodes *et al*. 2018).

Indeed, these complications can adversely affect multiple organs, contributing to an increase in years lost and premature death (Thomas 2022). Indeed, to mitigate the risk of complications linked to diabetes, maintaining optimal glycemic control with an HbA1c level below 7% has proven to be essential(Jefferies, Rhodes *et al*. 2018). In an Iraqi study, it was found that not only is there an increasing prevalence of type 2 diabetes mellitus (T2DM), but also the glycemic control among those with T2DM is suboptimal. For instance, in Basra, Iraq, a study reported that 86% of patients exhibited poor glycemic control, as evidenced by an HbA1c level exceeding 7% (Mansour, Alibrahim *et al*. 2020)**.**

1.1.1.3 Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is a condition characterized by elevated blood sugar levels and is initially detected during pregnancy, making it the most prevalent medical complication during this period. GDM affects approximately 15% of pregnancies worldwide, contributing to approximately 18 million births per year. Women with GDM face risk factors such as gestational hypertension, pre-eclampsia, and an increased likelihood of cesarean section. In addition, GDM increases the risk of complications such as cardiovascular disease, obesity, and carbohydrate malabsorption, ultimately increasing the risk of incident diabetes in both mother and

baby so two (T2DM) is greater. The increasing incidence of GDM poses a significant financial burden, underscoring the need for increased attention and awareness (Modzelewski, Stefanowicz-Rutkowska *et al*. 2022).

According to the most recent statistics provided by the International Diabetes Federation (IDF), gestational diabetes mellitus (GDM) affects 14.0% of pregnancies worldwide, with a 95% confidence interval between 13.97% and 14.04%. This number corresponds about 20 million births per year (Wang, Li *et al*. 2022). Women diagnosed with gestational diabetes mellitus (GDM) have an increased risk of gestational hypertension, pre-eclampsia and surgical cesarean section (Kondracki, Valente *et al*. 2022).

Gestational diabetes mellitus (GDM) is characterized by the development of glucose intolerance during pregnancy, which represents the most common complication during this period. A mainstay of treatment for GDM is dietary intervention, with pharmacologic interventions when necessary. If left untreated, GDM is associated with a high risk of adverse outcomes for the mother and fetus during pregnancy and delivery, including macrosomia, polyhydramnios, and the possibility of caesarean delivery(Shindo, Aoki *et al*. 2021). Also, the risk of maternal and neonatal birth trauma is increased, especially for shoulder tenderness, neonatal hypoglycemia, and prolonged pneumonia (Wicklow and Retnakaran 2023). More than , GDM is associated with long term adverse effects in postpartum women, such as an increased risk of diabetes mellitus, and in newborns, infants are more susceptible to adult metabolic syndrome (Wicklow and Retnakaran 2023).

1.1.2 Pathophysiology

The precise cause of hyperglycemia in patients with lean type 2 diabetes mellitus (T2DM) is not fully understood. A prominent theory gaining traction is the concept of sarcopenic obesity, where metabolic obesity results from excess adiposity accompanied by reduced muscle mass. In this scenario, beta cells that are already compromised struggle to handle even the minimal insulin resistance associated with a lean body weight. It seems reasonable to propose that the proportion of fat to total body weight is more crucial than the overall body weight itself. The crucial question arises: should the focus be on achieving a lower body weight or reducing body adiposity to prevent diabetes? This question is likely pivotal, although achieving this goal may present significant challenges (Baker, Overvad *et al*. 2019).

Moreover, there is conflicting evidence regarding the benefits of weight loss in this particular group of patients. In practical terms, the approach should be individualized. Some studies indicate a significant association between weight loss and a reduced risk of diabetes in both obese and non-obese patients. Conversely, other studies have reported adverse effects of weight loss, particularly in lean patients(Kim, Jeong *et al*. 2018). The primary pathophysiology seems to involve a swift failure of beta cells, likely attributed to a higher prevalence and early initiation of insulin use. Numerous studies have supported this hypothesis (Tsujimoto and Kajio 2019).

1.1.3 Epidemiology

Based on data from the International Diabetes Federation (IDF) for 2019, 463 million individuals between the ages of 20 and 79 had diabetes, and the disease was responsible for 4.2 million fatalities. This number is expected to rise sharply to approximately 700 million by2045. These epidemiological data demonstrate the wide spread and global impact of diabetes. Diabetes is the leading cause of at leaest $720 billion in health care(Wahiduzzaman 2021).

The global prevalence of diabetes represents an important and growing public health challenge. Recent estimates indicate that in 2021 there will be 537 million adults with diabetes, and by 2045 this number is projected to reach 783 million. This increasing trend underscores the urgent need to develop comprehensive strategies and interventions to address the impact of diabetes and reduce impact globally(Forouhi and Wareham 2022).

The burden of diabetes is unevenly distributed, with three-quarters of people with diabetes living in low- and middle-income countries. Factors such as population growth, worldwide prevalence of obesity, and unhealthy health practices including lack of nutrition and physical activity contribute to this burden. Diabetes mellitus is divided into two main categories: type 1 and 2, with type 2 diabetes accounting for more than 90% of the overall prevalence Both types can cause complications affecting multiple systems, a microvascular endpoint (such as retinopathy, nephropathy, neuropathy) are included and large vessel endings (such as the coronary heart disease, stroke, and peripheral neuropathy). The role of modifiable factors in the pathogenesis of type 2 diabetes is well understood, making prevention a realistic and important public health goal(Rosenfeld, Kelly et al. 2022).

1.1.4 Treatment of Diabetes Mellitus

1.1.4.1 Non-Pharmacological Treatment

1.1.4.1.1 physical alternations or life style changes

Targeted behavioral changes using lifestyle changes—such as weight loss and increased physical activity—have been shown to be beneficial in controlling blood pressure, cholesterol, and glycemic indices are reduced but it is important to emphasize that other preventive measures are also needed. Control of blood pressure and reduction of cholesterol levels are not only important for blood sugar control, but also play an important role in reducing cardiovascular risk factors and other related complications. A comprehensive approach incorporating lifestyle changes and medical interventions is essential to effectively manage diabetes and reduce the risk of complications (Cannata, Vadalà *et al*. 2020) .

1.1.4.1.2 dietary modifications

Evidence from randomized controlled trials conducted in the US. and Europe shows that lifestyle changes such as regular exercise and dietary advice can significantly reduce the incidence of diabetes in high-risk populations by 58% over three to five years. For individuals with prediabetes, the American Diabetes Association (ADA) supports comprehensive behavioral and lifestyle intervention programs. It is advised to engage in moderate-intense physical activity for at least 150 minutes a week in order to attain and maintain a 7% reduction in starting body weight. These recommendations emphasize how crucial proactive lifestyle modifications are for managing and preventing diabetes (Aweko 2019).

1.1.4.2 Pharmacological Treatment of T2DM

The first pharmacological treatment of T2DM came from insulin isolated from an animal pancreas in the 1920s. In recent years, many orally administered agents and injectable drugs have been developed to treat patients with T2DM. They can be used individually and/or in combination. These medications are decreased blood glucose levels, reducing body weight and the risk of cardiovascular complications (Deepthi, Sowjanya et al. 2017). However, there are differences in the effectiveness of medications for weight reduction, with results ranging from little weight loss (less than 3.2% of starting weight) to significant weight loss (more than 5% of starting weight) (Lazzaroni, Nasr et al. 2021),types of anti-diabetic drugs include alpha-glucosidase inhibitors, insulin sensitizers (biguanides, thiazolidinediones), insulin secretagogues (sulphonylureas, meglitinides), and incretin-based therapies (glucagon-like peptide-1 receptor agonists, dipeptidyl peptidase-4 inhibitors). Sodium-glucose cotransporter 2 inhibitors are another class of medication that lowers hyperglycemia by increasing the excretion of glucose in the urine (van Baar, van Ruiten et al. 2018).

1.1.5 Sodium Glucose Transports (SGLTs)

In individuals without diabetes, nearly all of the filtered glucose at the glomerulus (approximately 160–180 grams per day) is effectively reabsorbed, resulting in the absence of glucose in the urine (Vallon 2020) , Nevertheless, once the plasma glucose surpasses a threshold level (approximately 180 mg/dl), urinary glucose excretion begins to rise proportionally with the elevation in plasma glucose concentration. It's important to note that this threshold for glycosuria tends to increase in patients with type 2 diabetes mellitus (T2DM) (Cowie and Fisher 2020).

The movement of glucose through epithelial cells, occurs in the kidney and the gut and is dependent upon active transport systems. In order to absorb glucose, energy from the sodium gradient across the brush-border membrane is used against the concentration gradient. This sodium gradient is easily maintained due to the Na+/K+ ATPase (Cowie and Fisher 2020). Glucose absorbers, sodium-glucose transporters (SGLTs), These proteins, which are members of a basic membrane protein family, were crucial to the 19th century's movement of nutrients such as glucose, amino acids, vitamins, osmolytes, and certain ions (Neumiller, White et al. 2010). Intestinal SGLT1 was first characterized in 1987(Hediger, Coady et al. 1987). This transporter, which is able to inhibit glucose metabolism, is found in the kidney, placenta, lung, testis, prostate, heart, skeletal muscle, hippocampus, and other parts of the brain (Pliszka and Szablewski 2021).

SGLT2 is a unique sodium-glucose cotransporter (SGLT) found predominantly in the epithelium of renal segment 1 of the proximal winding duct SGLT2 has low affinity for glucose but high potency. Its primary function is to resorb more than 90% of the glucose filtered in the glomerulus (Sano, Shinozaki et al. 2020). Although SGLT2 is found predominantly in the kidney, especially in the epithelium of the proximal winding duct of kidney segment 1, it is also found in the brain, liver, thyroid, and skeletal muscle, although at much lower levels. With the help of SGLT1, any glucose in the tubule that is not absorbed by SGLT2 is reabsorbed in the more distal segments 2 and 3 of the proximal convoluted tubule and proximal straight tubule, respectively(Cowie and Fisher 2020). When SGLT2 is pharmacologically blocked almost entirely, only around 50–60% of filtered glucose is excreted, as opposed to the usual >90%. This decline is explained by an increase in SGLT1-mediated transport, which makes up for SGLT2's reduced activity(Rieg, Masuda et al. 2014). SGLT3 is another member of the Sodium-Glucose

Cotransporter (SGLT) family. It is expressed extensively in the body and seems to function as a glucose sensor. Moreover, SGLT4, SGLT5, and SGLT6 are known to exist, although nothing is known about their functions in humans (Cowie and Fisher 2020).

Inhibitors of the sodium–glucose cotransporter 2 (SGLT2) comprise a class of medications designed to lower blood glucose levels by blocking the renal reabsorption of glucose (Nespoux and Vallon 2018). In general, all of the released glucose is reabsorbed by the renal tubules, and SGLT2 accounts for approximately 90% of this reabsorption. Under hyperpurifying conditions, overexpression of SGLT2 results in increased reabsorption. SGLT2 inhibitors work by interfering with this reabsorption mechanism that increases glucose uptake into the urine (Moradi-Marjaneh, Paseban et al. 2019). The SGLT2 inhibitors currently in use are analogous to phlorizin, a naturally occurring compound extracted from the apple tree(Abbas, Al Harrasi et al. 2019). There are currently four phlorizin-based SGLT2 inhibitors commercially available in the US. and Europe(Moradi-Marjaneh, Paseban et al. 2019), Canagliflozin, dapagliflozin, empagliflozin, and ertugliflozin are four phlorizin-based SGLT2 inhibitors marketed in the United States and Europe. Also luseogliflozin and tofogliflozin have been approved in Japan. Not only do these drugs effectively lower blood sugar levels, they also show promise for improving heart health, reducing kidney problems and restoring cholesterol levels. It can be used as monotherapy or in combination with other antidiabetic agents to treat individuals with type 2 diabetes, especially when used with metformin(Tentolouris, Vlachakis *et al*. 2019).

Sodium-glucose cotransporter 2 (SGLT2) functions as a key cotransporter responsible for glucose reabsorption in the kidney. SGLT2 inhibition has become a

cutting-edge therapeutic approach for the treatment of diabetes. SGLT2i have demonstrated notable benefits in preventing heart failure (HF) and reducing cardiovascular mortality(Delgado, Jódar *et al*. 2022). This represents a significant milestone as the first evidence of anti-diabetic medications playing a preventive role in major cardiovascular events. Multiple large-scale trials have consistently yielded positive results regarding the prevention of heart failure through the use of SGLT2 inhibitors as in figure (1-1) (Hwang, Cho *et al*. 2020).

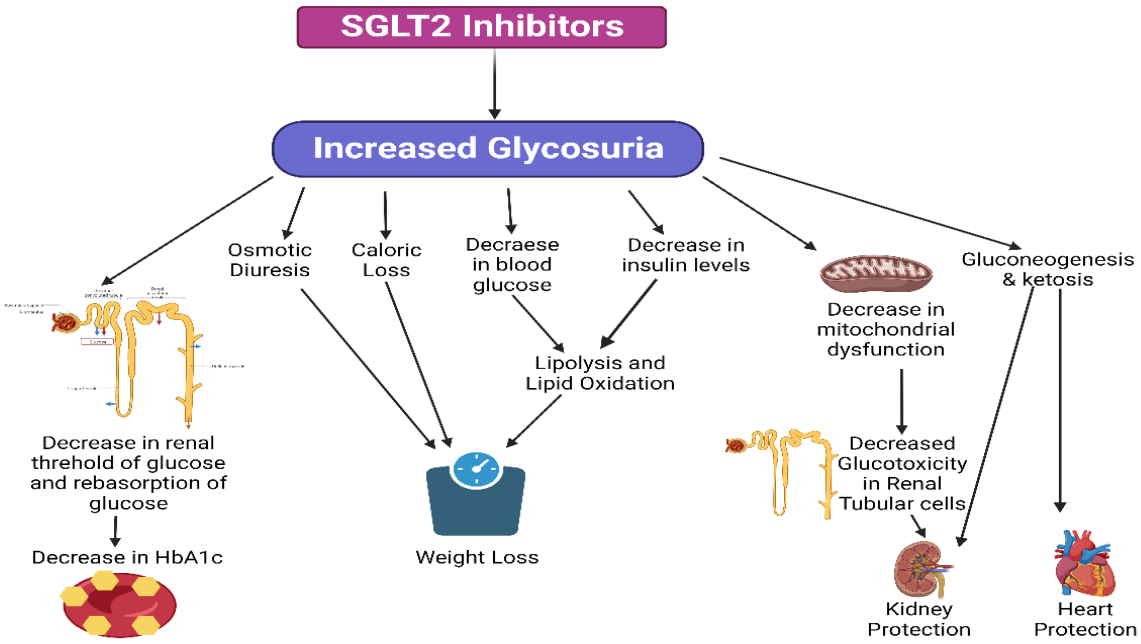

Figure (1-1): Mechanism by which SGLT2 inhibitors led to increased glycosuria and decreased HbA1c levels and body weight. Increased glycosuria leads to protective cardio-renal effects(Kaur, Kotru et al. 2023)

1.1.5.1 The Function of SGLT2 Inhibitors

A healthy person's glomeruli filter around 180 grams of glucose per day at normal glucose concentrations; almost all of the glucose is then reabsorption into the

bloodstream via the proximal tubule. Because of this, there is hardly any glucose in the urine (glycosuria) in this condition. About 90% of the tubular glucose load is actively reabsorbed with normal renal function, and the SGLT2 plasma membrane cotransporter helps to make this happen (Wright, Loo et al. 2011), Primarily located in the early section (S1 and S2 segments) of the renal tubule, SGLT2 has a low affinity and a high capacity for glucose. It makes it easier for sodium and glucose to be reabsorbed in a 2:1 ratio. About 90% of the glucose that has been filtered is efficiently reabsorbed by SGLT2. The SGLT1 transporter reabsorbs the final 10% of glucose that does not go through this pathway. SGLT1 is more prevalent in the gut and plays a critical function, especially in the absorption of glucose and galactose. It has a high affinity and limited capacity for glucose (Solini 2016).

GLUT2 facilitates the process of facilitated transport, which releases glucose passively at the basolateral membrane of the tubular cell and allows it to re-enter the circulation. Insulin activity is not necessary for the two transporters, SGLT2 and GLUT2, to function. Tubular reabsorption grows proportionately as prolonged hyperglycemia develops and the plasma glucose concentration rises, reaching its maximum at around 350 mg/min/1.73 m2, or 180–200 mg/dl of glucose. Any extra glucose that is consumed over this threshold exceeds the tubular reabsorption limit and is eliminated as urine.

In practice, there is no specific threshold beyond which this occurs, and the concept of "splay" refers to urinary glucose excretion that becomes noticeable before Tmax (maximum tubular reabsorption capacity) is reached. This variability can be attributed to the fact that different nephrons may release glucose at different thresholds, with some releasing at lower levels and others at higher levels. Additionally, the relatively low affinity of the glucose transporters contributes to this variability in glucose excretion (Ferrannini and Solini 2012). This concept is recalled in Figure (1.2)

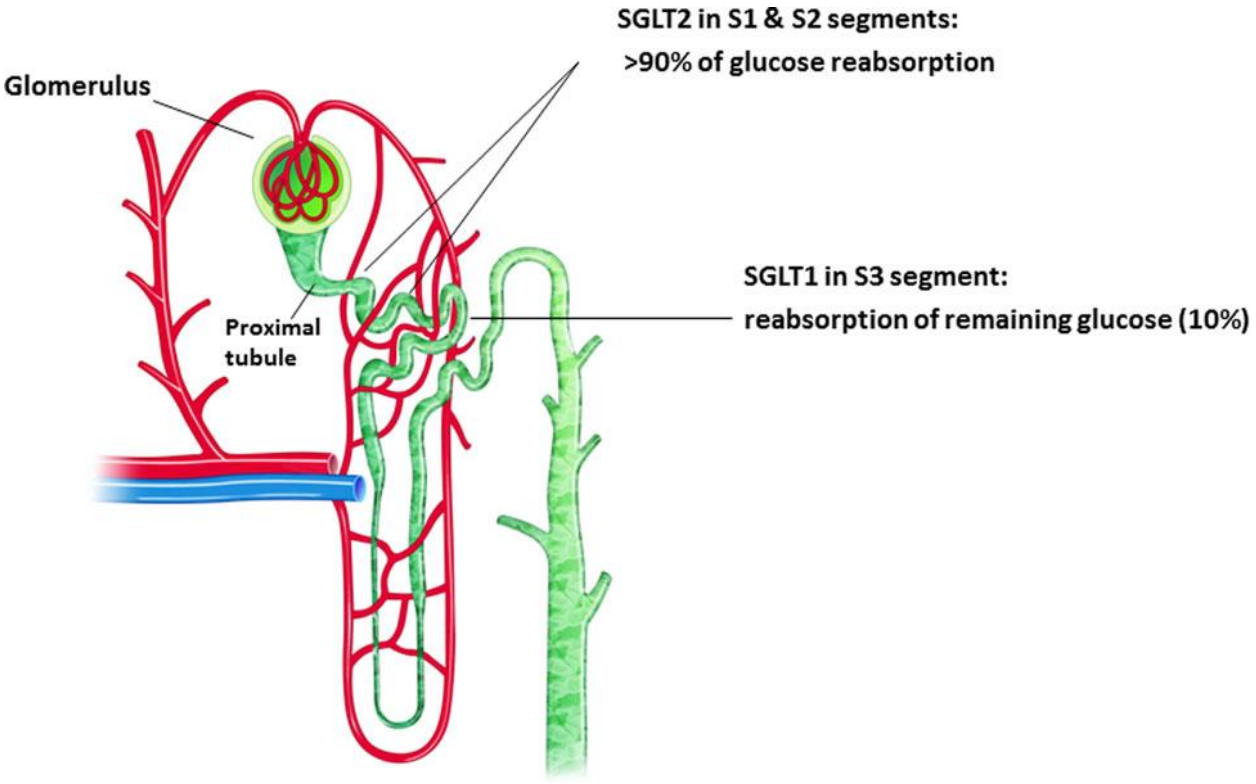

Figure (1-2): Glucose renal reabsorption through sodium–glucose cotransporters (SGLT1 and SGLT2) in the proximal tubule of the kidney. These transporters play a crucial role in reclaiming filtered glucose and preventing its excretion into the urine(Solini 2016)

The precursor to modern SGLT2 inhibitors was phlorizin, originally derived from the root of the apple tree (Ehrenkranz, Lewis et al. 2005), Over 20 years ago, Rossetti et al (1987) that the potential of phlorizin as a treatment for diabetes was effectively demonstrated in experiments involving partially pancreasectomized rats(Rossetti, Smith et al. 1987). The administration of phlorizin led to a noteworthy improvement in glucose levels and insulin resistance. Despite these promising results, further experimental work on this compound was discontinued due to its

restricted bioavailability after oral administration, notable gastrointestinal side effects, and concerns about potential interference with brain transporters.

Research done in the 1980s revealed that phlorizin-induced glycosuria was associated with the normalization of plasma glucose levels without resulting in hypoglycemia in a rat model of type 1 diabetes mellitus. In control animals with an undamaged pancreas, it had no effect on insulin action, but it was connected with the restoration of insulin sensitivity in rats that had part of their pancreas removed. It's interesting to note that insulin resistance and hyperglycemia returned when phlorizin medication was stopped (Rossetti, Smith et al. 1987). Phlorizin's strong GLUT1 inhibition and low oral bioavailability made it unsuitable for clinical development (Ehrenkranz, Lewis et al. 2005). As a result, research in pharmaceuticals shifted its attention to the creation of derivatives of phlorizin that have better bioavailability, stability, and selectivity for SGLT2 rather than SGLT1.

Many of these drugs have advanced to get clearance for marketing, and four of them—canagliflozin, dapagliflozin, empagliflozin, and ertugliflozin—are now approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

In addition, Japan has approved the use of three more SGLT2 inhibitors: tofogliflozin, luseogliflozin, and ipragliflozin. Remogaliflozin has been given the all-clear for clinical usage in India. Notably, the dual SGLT1 and SGLT2 inhibitor sotagliflozin has not yet been approved for clinical use in patients with type 1 diabetes mellitus in Europe, despite receiving permission for use in this population(Hropot, Battelino et al. 2023).

1.1.6 Genetics Variations (polymorphism)

Genetic polymorphisms, with a frequency of 1% or more, are more common than mutations among varied individuals. Numerous genetic variants, such as insertions, repetitions of certain sequences, and deletions, may be found in the

human genome. One of the most common types of genetic diversity is found in single nucleotide polymorphisms, or SNP(Albert 2011, Jin, Wang et al. 2018).

Single nucleotide polymorphisms are variations in a genetic sequence that affect only one of the basic building blocks of a DNA molecule, cytosine (C), guanine (G), adenine (A),thymine (T) or SNPs. More than 1% of all SNPs are found in the population, and two-thirds of them include thymine substitution for cytosine (C). These differences add to the genetic diversity among individuals (Robert and Pelletier 2018, Chen, Huang et al. 2021)

Single Nucleotide Polymorphisms (SNPs) account for roughly 90% of all human genetic diversity, with one occurring for every 100-300 nucleotides inside the human genetic code. SNPs are present in both the non-coding (intron) and coding (exon) regions of the genome. SNPs in coding regions are further divided into nonsynonymous and synonymous groups. Synonymous SNPs have no effect on the function of the protein or alter its amino acid sequence. Two types of nonsynonymous SNPs are distinguished: absurdity, which causes premature termination of protein synthesis, and missense, which results in a change in the protein's amino acid sequence. These variants have a substantial role in the genetic diversity found among people(Chu and Wei 2019, Conforti, Tuettelmann et al. 2022).

Missense SNPs cause amino acid alterations that have a deleterious effect on protein structure and/or function, leading to disease development and influencing physiological medication responses. On the other side, nonsense point mutations are DNA sequence alterations that result in the insertion of a stop codon, resulting in a nonfunctional protein product(Ismail and Essawi 2012, Ramírez-Bello and Jiménez-Morales 2017).

1.1.6.1 Role of genetic variation in the human sodium–glucose cotransporter 2 gene (SGLT2) in glucose homeostasis

genetic factors play an important role in the risk of development late T2D complications (Klen, Goričar et al. 2015, Klen, Goričar et al. 2015). Unlike single-gene inherited disorders that follow a straightforward pattern, Type 2 Diabetes (T2D) is a multifactorial disease with a complex origin. Its development involves a combination of genetic and environmental factors, playing significant roles in the clinical condition and pathology. Genetic factors are considered pivotal predisposing elements influencing individual susceptibility to T2D. Therefore, the identification of these predisposing genetic variants becomes a crucial step in managing T2D, potentially improving the clinical condition and preventing complications. Individuals can benefit from personalized therapies by understanding the specific genetic and environmental factors that contribute to the onset of this chronic disease (Sirdah and Reading 2020). SGLT2 shows significant potential for preventing renal, cardiovascular, and neurological complications in individuals with type 2 diabetes (Vasquez-Rios and Nadkarni 2020).

SGLT2, encoded by the SGLT2 gene also identified as SLC5A2 (soluble carrier family 5 member 2), is located on chromosome 16. SLC5A2 gene mutations, which affect SGLT2 expression, membrane localization, or transporter the activity is associated with the family concept glucosuria diarrhea. This condition results in abnormally high urinary glucose excretion despite normal blood glucose levels(Klen and Dolžan 2021) . In our study of empagliflozin in patients with diabetes, this drug belongs to the class of sodium-glucose transporters 2 (SGLT2) inhibitors. It functions by inhibiting sodium and glucose transporters in the kidneys.

1.1.7 Empagliflozin

The pharmaceutical landscape has recently included empagliflozin, which inhibits sodium-glucose cotransporter 2 (SGLT2). It lowers glucotoxicity and insulin resistance and improves glucose metabolism and glycemic management by increasing urine glucose excretion. Empagliflozin was first created to treat hyperglycemia in people with type 2 diabetes mellitus (T2DM), but research has since revealed that it also has a number of other benefits. It is a breakthrough in the treatment of heart failure (HF) and has nephroprotective characteristics. Notably, studies have indicated that empagliflozin reduces the number of heart failure hospitalizations as well as the mortality rate from cardiovascular causes (Packer, Anker et al. 2020)

Empagliflozin administered as monotherapy or as an adjuvant to treatment successfully decreases average daily glucose levels, glycated hemoglobin A1C (HbA1C), postprandial blood glucose, fasting blood glucose, and causes considerable weight reduction in people with type 2 diabetes. This is especially beneficial for patients with suboptimal glycemic control (Forycka, Hajdys et al. 2022).

Type 2 diabetes mellitus (T2DM) may now be treated with medications, and a key transporter for the reabsorption of glucose from the glomerular filtrate is the sodium-glucose cotransporter 2 (SGLT2). It is a new and important goal for the management of type 2 diabetes (Chawla and Chaudhary 2019). By effectively blocking glucose reabsorption in the proximal tubule, it leads to an increased excretion of glucose in the urine (Chatterjee, Khunti et al. 2016, Wanner, Lachin et al. 2018).

Empagliflozin acts selectively on SGLT2 transporters(Wanner, Lachin et al. 2018). This is important because the proximal tubule segments express SGLT2 (Vallon and Thomson 2020), where 90% of glucose reabsorption occurs(Silva dos Santos, Polidoro et al. 2020) .On the contrary, SGLT1 can be expressed in the intestines, heart, skeletal muscle (Chadt and Al-Hasani 2020).This leads to glucosuria and a decrease in plasma glucose levels (Wanner, Lachin et al. 2018). Furthermore, it has been shown that empagliflozin lessens oxidative stress. When this drug is administered, it decreases the action of prooxidant factors, prevents the generation of reactive oxygen species (ROS), and enhances mitochondrial function (Andreadi, Bellia et al. 2022).

When treating type 2 diabetic mellitus (T2DM), the first dosage of empagliflozin is 10 mg, either orally as a monotherapy or in combination. Because the mechanism of SGLT2 inhibition is independent of insulin sensitivity or circulating insulin levels, these medications can be used in conjunction with exogenous insulin and all other antidiabetic classes. Insulin may be provided at a lower dose to reduce the risk of hypoglycemia when combined with a sulfonylurea. For better glucose control, patients taking 10 mg of empagliflozin once daily and having an estimated glomerular filtration rate (eGFR) greater than 60 mL/min/1.73 m^2 may raise the dosage to a maximum of 25 mg daily.

Because of how it works, renal function affects how effective empagliflozin is in lowering blood sugar levels in individuals with type 2 diabetes. In those with moderate renal impairment, its efficacy is reduced, and in those with severe renal impairment, it could not exist at all. Several anti-hyperglycemic medications should be taken into consideration if individuals with T2DM and concomitant renal impairment need further glycemic control. In the event that the eGFR is less than 30

mL/min/1.73 m², empagliflozin should not be administered(Forycka, Hajdys et al. 2022).

1.1.8 Aims of study

1.To explore the existence and impact of genetic polymorphism in SGLT2 (sodium-glucose cotransporter 2) among individuals with type 2 diabetes mellitus in various provinces of Iraq.

2. To examine the influence of genetic polymorphism in SGLT2 (sodium-glucose cotransporter 2) on the therapeutic response to empagliflozin among individuals diagnosed with type 2 diabetes mellitus in different provinces of Iraq.

Chapter Two

Subject, Materials and Methods

2. Subject, material and methods

2.1 Subjects: Control and Patients

There were 110 participants in the study, ranging in age from 29 to 73. During their appointments at a private clinic for medical care and counseling on personal issues, participants were enlisted. The study was conducted from November 2022 to May 2023.

According to accepted diagnostic standards, type 2 diabetes mellitus had previously been identified in each of the patients. In addition, 50 healthy people in the age range of 30 to 50 were included as a comparison control group.

2.1.1 Patients Criteria

2.1.1.1 Inclusion Criteria

The patients diagnosed with type 2 diabetes mellitus (T2DM) were undergoing a treatment regimen of 10 mg/day empagliflozin for a duration ranging from 6 months to 2 years. These individuals did not have any other coexisting diseases.

2.1.1.2 Exclusion Criteria

1. The subjects included in the study with other therapy involving insulin and insulin secretagogues or other medications.

2. Patients with severe renal failure, malignant illnesses, autoimmune disorders, chronic hepatic problems, or hypothyroidism were not included in the study.

2.1.2 Ethical and Scientific Approval

 The research proposal was thoroughly examined and approved by the scientific and ethical committee at the College of Pharmacy, Karbala University.

 All subjects actively participated in the study after obtaining informed permission. Each participant completed the permission form, which provided a full description of the study's aims. Participants were invited to complete a specifically created questionnaire.

2.1.3 Study Design

This study, which includes 110 Iraqi people with type 2 diabetes, is cross-sectional in nature. As a control group, 50 individuals who seemed healthy and had no known diseases were also included. Blood samples were collected for biochemical, hormonal, and genetic research purposes from fasting participants who had previously taken empagliflozin.

2.2 Materials

2.2.1 Kits, Chemicals, and Providers

The adjacent table lists the chemicals and kits utilized in this inquiry along with the corresponding production companies (2-1).

Table (2-1): - Instruments and the manufacturing companies

	Chemicals and Kits	Company	Country
Chemicals	Agarose	Bio Basic	Canada
	Ethanol	Hayman Kimia	UK
	Ethediem Bromide	Intron	Korea
	Nucleasfree water	Bioneer	Korea
	TBEbuffer	Bioneer	Korea
Biochemical Kits	CholesterolKit	Mindray	China
	Fasting serum glucose kit	Roche	Germany
	Glycosylated Hemoglobin kit	Minday	China
	Insulin kit	Mindray	China
	Triglyceride kit	Mindray	China
Kits For Genetic Study	DNA extraction kit	Intron	Korea
	DNA ladder marker	Intron	Korea
	PCR PreMix Kit	Bioneer	Korea
	Primers	Bioneer	Korea

2.3 Methods

2.3.1 Samples Collections

Blood samples 5 ml were obtained from both patients and healthy controls following an overnight fast. Two groups were created from the blood samples that were obtained. In order to extract DNA, the first half (2 ml) was kept in an EDTA tube, while the second portion (3 ml) was kept in a gel tube to aid in the isolation of serum for ensuing biochemical studies.

2.3.2 Methods of Biochemical Assays

2.3.2.1 Determination of Glycemic Indices

2.3.2.1.1 Estimation of Fasting Serum Glucose

In this investigation, the following guidelines were applied to calculate the fasting serum glucose (FSG): Hexokinase is an enzyme that helps phosphorylate glucose to glucose-6-phosphate by utilizing ATP. Next, using UV in the presence of NADP, the glucose level is ascertained. Gluconate-6-phosphate is produced when glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase. Crucially, this process does not result in the oxidation of any more carbohydrates. Photometric measurements show that the rate at which NADPH is formed during the reaction is closely related to the concentration of glucose(Schlenker 1997).

2.3.2.1.2 Estimation of Fasting Serum Insulin

The CL-series insulin test, a two-site immunoenzymatic assay, was utilized in this investigation to measure insulin levels. The sample, paramagnetic microparticles coated with monoclonal anti-insulin antibody, and an alkaline phosphatase-monoclonal anti-insulin antibody conjugate are added to a reaction cuvette in the first phase. Insulin in the sample forms a sandwich complex during incubation when it binds to the anti-insulin antibody alkaline phosphatase conjugate and the anti-insulin antibody-coated microparticles. The unattached chemicals are removed by washing after incubation, and the microparticles are magnetically retained.

The system's photomultiplier calculates the chemiluminescent reaction in terms of relative light units. The quantity of light units produced during the reaction is directly correlated with the amount of insulin present in the sample (Bürgi, Briner et al. 1988).

2.3.2.1.3 Estimation of Glycosylated Hemoglobin

Using the BS240Pro apparatus, a blood sample is automatically hemolyzed in an EDTA tube to determine glycosylated hemoglobin (HbA1c). This method eliminates leukocyte interference by using the detergent tetradecyltrimethylammonium bromide (TTAB) in the hemolyzing reagent. The levels of antibody-recognizable regions, which are similar to those detected in HbA1c, are measured by this test and are present in all glycated hemoglobin at the beta-chain N terminal.

A soluble antigen-antibody complex is produced in the sample as a result of glycohemoglobin and anti-HbA1c antibody interaction. Since the HbA1c antibody

site is found only once in HbA1c molecules, this combination is not possible. Turbidimetry can be used to detect insoluble antibody-polyhapten complexes formed when excess anti-HbA1c antibody binds to polyhapten (Weykamp, 2013)

Estimated average glucose (eAG) was calculated as an average based on HbA1C readings and expressed as mg/dL against %. This was determined using the following formula: eAG = 28.7 times HbA1C - 46.7(Ebid, Ehab et al. 2019)

2.3.2.2 Determination of Lipid Profile

2.3.2.2.1 Estimation of Total Cholesterol

Serum total cholesterol (TC) was measured using the BS240Pro in vitro system using an enzymatic colorimetric technique. The enzyme cholesterol esterase (CE) converts fatty esters into fatty acids and unsaturated fatty acids. Fats are then oxidized by the enzyme cholesterol oxidase (CHOD), which generates hydrogen peroxide and cholest-4-en-3-one. The peroxidase enzyme (POD) then uses hydrogen peroxide to catalyze the oxidative coupling of phenol and 4-aminoantipyrine (4-AAP) producing a red quinone-imine dye.

The intense color of the solution, which shows an increase in absorbance at 512nm, is directly related to the lipid content (Hopkins 2004).

2.3.2.2.2 Estimation of Serum Triglyceride

Using the BS240Pro system, an enzymatic colorimetric method was used to determine the blood triglyceride level. In this process, triglycerides are hydrolyzed by lipase to produce glycerol. Glycerol kinase and glycerol-3-phosphate oxidase then convert glycerol to H2O2. Using a color-producing method catalyzed by

peroxidase, the amount of H2O2 generated is calculated, and the absorbance is measured at 500 nm. The amount of triglycerides in the color corresponds to its intensity(Hopkins 2004)

triglycerides + 3 H_2O $\xrightarrow{\text{Lipoprotein lipase}}$ glycerol + 3 RCOOH

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

glycerol-3-phosphate + O2 $\xrightarrow{\text{Glycerol-3-Phosphate Oxidase}}$ dihydroxyacetone phosphate + H_2O_2

H_2O_2 + 4-aminophenazone + 4-chlorophenol $\xrightarrow{\text{peroxidase}}$

4-(p-benzoquinone-monoimino)-phenazone + 2 H2O + HCl

2.3.2.3 Determination of Body Mass Index

The Body Mass Index (BMI) is a metric calculated by dividing an individual's body weight (in kilograms) by the square of their height (in meters). This calculation results in a unit of measurement expressed as kilograms per square meter (kg/m2). The BMI is commonly used to assess body weight in relation to height(Rahman and Berenson 2010)

BMI=Weight /(Height)2

BMI readings between 18.5-24.9 denote normal weight, readings between 25 and 30 denote overweight, while readings over 30 denote obesity.

2.3.2.4 Determination renal function

2.3.2.4.1 Estimation of serum creatinine

Creatinine Blood Test: is a test that measures the level of creatinine in the blood, which is a kidney function test. Creatinine is known as a waste product made by the muscles, and the kidneys in the individual's body usually get rid of the creatinine in

the blood and excrete it outside the body through urine. A problem with the urine leads to an increase in the amount of creatinine in the blood, while its amount in the urine decreases. a high creatinine test in the blood does not always indicate the presence of a health problem in the individual, but a high level of creatinine in the blood can sometimes indicate the presence of some diseases, such as: Kidney, hyperthyroidism, or bacterial infection.

Creatinine blood tests are usually performed along with several other laboratory tests, including a blood urea nitrogen (BUN) test and a basic metabolic panel (BMP) or comprehensive metabolic panel (CMP). These tests are done during routine physical exams to help diagnose certain diseases and to check for any problems with your kidney function.

Principle

The Jaffe reaction involves the reaction of creatinine with picric acid in an alkaline solution, resulting in the formation of a reddish-colored complex known as the Janovski complex.

Materials

- 16 x 100 mm Test Tubes
- Creatinine Standard
- Picric Acid Reagent
- Controls
- Sodium tungstate
- Patient Samples
- Sulfuric Acid
- Spectrophotometer
- Distilled Water
- Sodium Hydroxide
- Table Top Centrifuge

Procedure

1. Mark test tubes measuring 16 by 100 mm for every patient sample and control being examined.

2. Add 1 mL of sulfuric acid reagent, 1 mL of sodium tungstate reagent, and 1 mL of distilled water to each patient sample tube and control tube. Stir well.

3. Fill the suitable tube with 1 mL of either patient or control serum.

4. Combine well and centrifuge at 1500 RPM for 5 minutes.

5. Mark clean 16 x 100 mm test tubes as sample, standard, blank, or control in order to prepare a "filtrate".

6. Use the reagents and samples to fill each tube. NaOH and the picric acid reagent can be added to each tube after the other ingredients have been added. Each test tube need to have the same finalMix thoroughly and let stand for 15 minutes at room temperature.

7.Transfer the contents of test tube to an appropriate cuvette and read the absorbance at 510 nm against the blank solution.

8.Record the results on the data sheet.

9.Determine the creatinine values for the control and patient samples by proportional calculation using the concentration of the standard and its absorbance.

2.3.2.4.2 Estimation of serum sodium

A sodium test is a medical test used to determine the sodium level in a person's blood or other body fluid. The test is used to diagnose and monitor certain diseases and conditions, such as high blood pressure and heart disease. A sodium test typically involves collecting blood or other body fluid samples for testing. Sodium levels in the bloodstream are affected by many factors, including diet, exercise, medical conditions, and medications. A sodium blood test can help diagnose and monitor these conditions.

Principle for Sodium:

Sodium and proteins are simultaneously precipitated using a reagent containing magnesium uranyl acetate with alcohol. After centrifugation, the precipitate is separated. The sodium content is determined by calculating the loss in the concentration of magnesium uranyl acetate in the reagent solution, compared to a standard sodium solution treated similarly. The remaining amount of magnesium uranyl acetate is assessed by forming brown (dark) ferrous uranyl acetate, which is then measured using a colorimeter.

Reagents:

1. A 33 ml of sodium precipitating reagent.
2. Standard 3 ml of Sodium and Potassium.

3. A10 ml of Sodium Color Reagent.

4. A 45 ml of potassium reagent.

When kept at 2-8°C, the reagents are ready to use and useable until the expiration date, assuming no contamination.

Sodium Assay:

Step I: Proteins and sodium precipitate.

Pipette into two dry, clean test tubes with the labels test (T) and standard (S).

	S	T
Sodium PPT Reagent(l)	1.0 ml	1.0 ml
Standard Sodium/Potassium (2)	0.02 ml	....
Serum	.....	0.02 ml

Stir well for one minute in a vortex, then let sit at room temperature for five minutes. Centrifuge at 3000 RPM for a minute.

Step II - Color Development.

Pipette into three clean dry test tubes labelled blank (B), standard (S) and test (T)

	B	S	T
Distilled Water	3ml	3ml	3ml
Supernatant from step I	...	0.05ml	0.05ml
Sodium PPT Reagent (1)	0.05ml	....	....
Sodium Color Reagent (3)	0.2ml	0.2ml	0.2ml

Stir thoroughly and allow it stand at room temperature for five minutes. A photo colorimeter set to 540 nm should then be used to measure the absorbance of B, S, and T against distilled water in ten minutes.

Calculation:

Sodium in mmol/L =

Absorbance of B-T X 150 (Standard concentration)

Absorbance of B-S

2.3.2.4.3 Estimation of blood urea

In clinical biochemistry labs, the quantitative measurement of blood urea is standard procedure. The liver produces urea, which is the main nitrogen molecule in blood that isn't a protein. It is created as a consequence of the amino acid deamination processes that occur during the breakdown of proteins. Urine urea elimination is the main method of nitrogen excretion. Urine flows slowly during passive tubular reabsorption, even if it is filtered from the blood at the glomerulus.

Urea is hydrolyzed by urease, which produces carbon dioxide and ammonia. A green chromophore is formed when the generated ammonia reacts with sodium salicylate, alkaline hypochlorite, and sodium nitroprusside, a coupling agent. The amount of urea present in the sample is correlated with the color intensity.

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

$$NH_4 + \text{Salicylate} + NaClO \xrightarrow{\text{Nitroprusside}} \text{Indophenol} + NaCl$$

Reagents

1. Urease (> 500 U/mL) is the enzyme reagent (Vial R1).

2. Buffered Chromogen (Vial R2): sodium salicylate (60 mmol/L), sodium nitroprusside (3.4 mmol/L), EDTA (2 mmol/L), and phosphate buffer (20 mmol/L, PH=6.9).

3. Sodium hypochlorite (10 mmol/L) and sodium hydroxide (150 mmol/L) are present in Alkaline Hypochlorite (Vial R3).

4-Urea solution at 50 mg/dL, the usual concentration. Preparing Reagents The contents of Vial R1 and R2 were combined and dissolved to create the Working Reagent Solution, which was then kept at 2 to 8°C and out of direct sunlight.

Procedure

1. Take patient blood samples, place them in serum blood tubes, and wait 20 minutes for the blood to coagulate.

2. Centrifuge a sample of serum at 3000–4000 RPM for 10–20 minutes to extract it from the blood.

3. Gather the serum into containers with labels.

4- Three tube sets were ready.

5. After vortexing each tube, they were incubated for either 10 minutes at 16–25°C or 5 minutes at 37ºC.

6. Fill each tube with 1 mL of R3 solution.

7. After vortexing each tube, they were incubated for five minutes at 37℃ or ten minutes at 16 to 25 ℃.

8- Using a cuvette with a 1 cm light path on the Sepctrophotometer, the absorbance (A) of the serum samples and standard was determined at wave length 600 nm in comparison to the blank.

2.3.3 Genetic Analysis

2.3.3.1 The selection of SLC5A2 gene SNPs

The SNPs investigated in this study, namely rs138803748 and rs121918621, were chosen based on information from the National Center for Biotechnology Information (NCBI). These specific SNPs were identified as the most prevalent in the SLC5A2 gene, as detailed in Table (2-2).

Table (2-2): - SNPs, Gene, Nucleotide change, Amino acid substitution that involved in current study.

SNPs	Gene	Nucleotide change	Amino acid substitution	Consequences
rs138803748	**SLC5A2**	**568G>A**	**Val190Met**	**Missense variant**
rs121918621	**SLC5A2**	**320G>A**	**Try440***	**Non sense**

Val: amino acid Valline; Met: amino acid methionine; Try: amino acid tryptophan; * Stop codon

2.3.3.2 Primer sequences of SLC5A2 rs121918621 and rs138803748 as shown in table (2.3) & (2.4)

Table 2-3**:** Primer sequences of SLC5A2 rs121918621

	Primer	**Primer sqaunce**	**PCR product**
Primer sequences of SLC5A2 rs121918621	**Forward Allele G**	**5-ATCGTGGTAGTGTCGGTGGCCTGG-3**	**400**
	Forward Allele A	**5-ATCGTGGTAGTGTCGGTGGCCTGA-3**	
	Reverse common	**5-TGAGGAGGCCAGAGCAGAAGAACA-3**	

Table 2-4: Primer sequences of SLC5A2 rs138803748

	Primer	**Primer sqaunce**	**PCR product**
Primer sequences of SLC5A2 rs138803748	**Forward Allele G**	**5-TTCTGGGCATCACCATGATTTACACGG-3**	**410 bp**
	Forward Allele A	**5-TTCTGGGCATCACCATGATTTACACGA-3**	
	Reverse common	**5-TACTCTGGTCCGCATCTGACCTCA-3**	

2.3.3.3 Extraction of Genomic DNA from Blood Sample

To facilitate the purification of total DNA from diverse biological samples, including blood, the Favor Prep Genomic DNA Mini kit by Favorgen was employed. This kit offers a rapid and straightforward method for obtaining pure DNA, making it suitable for both immediate use and storage.

1- RBC Lysis

1.Place 1.5 mL of whole blood in a microcentrifuge tube with 300 µl of blood.

2. Put 30µl of proteinase K enzyme in a microcentrifuge tube and pulse-vortex.

3. Invert the mixing process after adding 900 µl of 3X RBC lysis buffer.

4. The sample combination was incubated for ten minutes at room temperature. Make sure that after incubation, the sample combination becomes translucent and deeply crimson.

5. Centrifuge for five minutes at 3000 x g to extract the supernatant entirely.

6. Add100µl of RBC lysis buffer to the peillet in order to resuspend the cells.

2) The Lysis of Cells

7. Use a vortex to thoroughly mix the sample mixture after adding 200µl of FABG buffer.

8. The mixture was incubated at room temperature for ten minutes. Throughout the incubation period, the tube was replaced every 3 minutes.

9. The elution buffer was placed in a 70 ºC water bath.

2- DNA Binding

10. The material was thoroughly combined with 200µl of pure ethanol using a 10-second vortex.

11. A collecting tube held the FABG column. After adding the mixture to the FABG column, cover it, and centrifuge for one minute at 14,000 rpm. Take out a fresh sample and discard the filter.

3- Column Washing

12. After adding 400µl of W1 buffer, the FABG column was centrifuged at 14,000 rpm for 30 seconds. Once the filtrate was disposed of, the column was put back into the collecting tube.

13. Fill the FABG column with 600µl of Wash buffer, then centrifuge at 14,000 rpm for 30 seconds. Throw away the filtrate. To finish drying it out, the column was put back into the collecting tube and centrifuged for a further three minutes.

4- Elution

14. The dried FABG column should be placed in a fresh 1.5 ml microcentrifuge tube. To the membrane center, add 100 µl of warmed elution buffer or TE.

15. The FABG column was centrifuged for one minute at 14,000 rpm after being incubated for ten minutes in an incubator to extract DNA. The piece of DNA is kept at -20°C.

2.3.3.4 Allele Specific Polymerase Chain reaction

The SLC5A2 gene was amplified using polymerase chain reaction (PCR) at two specific loci, rs121918621 and rs138803748. Using primer-BLAST software, the primers utilized in this amplification were generated. They were then bought in lyophilized form at various concentrations in picomoles from Bioneer in Korea. Reconstituted primers were made into a stock solution of 100 pmol/µl using nuclease-free water. Ten microliters of each primer stock solution were combined with ninety microliters of nuclease-free water to form the working solution. Until it was required, the working solution was stored at -20°C. The SNP location (rs121918621) in the SLC5A2 gene is depicted in Figure (2-1)

GCCGGCGACCGCGAGCTGCTGCTGGTGGGACGGTGCGGCCTGGG
CTCCCCTCCTCCCCAACGGATCAGCCCGGGGCGGGGGCTTGCGC
ACCTGCAGGGGAGCCCAGGGTCCGGGTTCGATCCGACGGCCTCC
GCCGCAGGCTCTGGGTGGTGTTCATCGTGGTAGTGTCGGTGGCC
TG[G>A]CTTCCCGTGGTGCAGGCGGCACAGGGCGGGCAGCTCTT
CGATTACATCCAGGCAGTCTCTAGCTACCTGGCACCGCCCGTGT
CCGCCGTCTTCGTGCTGGCGCTCTTCGTGCCGCGCGTTAATGAGC
AGGTGAGCGGCACGCGCGTGGTGACGGCAGGGCTGGGCTTGCA
CATCCTCAGCAGGC

Figure (2-1): - SNP (rs121918621) of SLC5A2

The Position of SNP (rs138803748) of SLC5A2 as shown in figure (2-2)

CTGGGCAGGAGGTGGGCTGGGGACACTGCCCTGGGTCCTGACCT
GGCACTTGCTTCTCCCCCAAGGTGGACATGTTCTCCGGAGCTGTA
TTCATCCAGCAGGCTCTGGGCTGGAACATCTATGCCTCCGTCATC
GCGCTTCTGGGCATCACCATGATTTACACG[G>A]TGACAGGTGC
CAGCAGGGGCTTAGGAAAGGGAGTGGGCCTGGGACACTGCTGC
CAGCTGTGGCCAGGGTTTAGGGAGCTGCCAGAGGAAAGCAAGC
TGGAGAGGTCTGGAAGGGTGGTGTGGTCCAAGCAGGAGAAGGA
ACTAAATCTTTGGAGAGCAAAGGCTTCCTCTCTTTCCACTGTCTC
TTGGATTTTGACTGG

Figure (2-2): - SNP (rs138803748) of SLC5A2

2.3.3.4.1 Optimization of Polymerase Chain Reaction Conditions

The ideal DNA and primer concentrations, the number of amplification cycles for the allele-specific PCR reaction, and the appropriate annealing temperature were all determined in an effort to enhance the PCR reaction. The PCR reaction components for all amplified fragments and optimal PCR methods are displayed in Tables (2-4) and (2-5), respectively.

2.3.3.4.2 Polymerase Chain Reaction

The DNA solution and the PCR components were combined, and the ideal PCR procedures were followed to carry out the PCR reaction.

2.3.3.5 Agarose Gel Electrophoresis

1. To produce 1x TBE buffer (Tris-Borate-EDTA), combine 10ml of 10x TBE buffer with 90ml of distilled water. Then, dissolve 1.5g of agarose powder in 100ml of this mixture.

2. For a few minutes, place the solution on a hot plate.

3. Add 4 µl of ethidium bromide to the solution when it has cooled.

4. To create wells for loading PCR products, the comb was attached to the end of the tray.

5. The agarose gel was poured into the tray and allowed to solidify for 20 minutes at room temperature.

6. The comb was carefully taken out of the tray.

7. TBE buffer was added to the tray and it was put into an electrophoresis cell.

8. Fill one well with 5 µl of DNA ladder, and the other wells with 4 µl of each PCR product.

2.4 Statistical Analysis

After being converted to an electronic database and verified for accuracy, the study's participant data were processed, maintained, and examined using IBM's Statistical Package for the Social Sciences(SPSS) version 26.

Logistic analysis of the SNP yielded the odds ratio (OR), and95% conefidence intervales were used to evaluate the OR's significance in examining the response to treatment.

The bivariate correlation test was utilized to investigate the link between the SNP and the mean diabetes variables. The correlation coefficient (R) was calculated on a 0–1 scale, greater numbers indicating a more robust positive correlation. The direction of correlation was indicated by the sign of (R), where a positive (unsigned) R value indicated a direct link and a negative signed R value indicated an inverse correlation.

A significance criterion of ($P < 0.05$) was used to indicate a significant difference or relationship. The findings were then presented in tables and/or figures, accompanied

Chapter Three

Results

3. Results

3.1 Demographic Data for Control and Type2 Diabetic Patients

The table (3-1) displays demographic data for 110 diabetics and 50 healthy individuals (control group). The age range of the participants was 29–73 years, and the control group's mean ± SD was 41.78 ± 4.99, whereas the patients' mean ± SD was 42.89 ± 4.59. The age differences between the diabetes and the control group were not statistically significant. The BMI of the control group was 28.17 ± 3.26, whereas the patients' BMI was 33.18 ± 3.32. This research reveals that T2DM patients had a significantly higher BMI than the healthy control group.

Regarding family history, the results also reveal significant differences between the two groups. However, there were no significant differences between the study groups concerning occupation.

Table (3-1): Socio-demographic data of diabetic patients and control group.

Variables		Groups		p-value
		Control (n=50)	Diabetics (n=110)	
Age (y)		41.78 ± 4.99	42.89 ± 4.59	**< 0.064 [NS]**
BMI(kg/m^2)		28.17 ± 3.26	33.18 ± 3.32	**0.0072 [S]**
Family history	**Mother**	3 (6%)	19 (17.4%)	**X2=26.44 0.0001 [S]**
	Father	11 (22%)	20 (18%)	
	Both	14 (28%)	15 (13.6%)	
	Non	22 (44%)	56 (51%)	
Region	**Rural**	21 (42%)	40 (36.6%)	**X2= 0.894 0.68 [NS]**
	Urban	29 (58%)	70 (63.4%)	
Results are presented as mean ± SD, n= number of subjects and% percentage , p<0.05 considered significantly different, [S]= Significant, [NS]= non-significant				

3.2 Assessment of Metabolic Parameters for Control Group and Type2 Diabetes Mellitus Group.

3.2.1 Glycemic Parameters of Healthy Control Group and Type2 Diabetic Patients Group.

Table (3-2) presents significant variations in all glycemic indicators, including glycated hemoglobin (HbA1c) and fasting blood sugar (FBG), and fasting serum insulin (FSI) between the research groups. FBG and HbA1c values in diabetes individuals were substantially higher than in the healthy group. Furthermore, fasting serum insulin (FSI) levels in diabetes patients were considerably lower than in the healthy group.

Table (3-2): Glycemic parameters of the healthy control group and type2 diabetic group.

Vareables	**Groups**		**p-value**
	Control(n=50) mean ±SD	Diabetics(n=110) mean ±SD	
FBG (mg/dl)	**117± 42.33**	**183.55 ± 45.02**	**0.032 [S]**
HBA1C	**4.21 ± 0.11**	**8.223 ± 1.42**	**< 0.027 [S]**
FSI (mg/dl)	**19.45± 5.73**	**13.7 ± 3.58**	**< 0.001[S]**
Results are presented as mean ± SD, p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant			

3.2.2 Lipid Profile of Healthy Control Group and type2 Diabetic Patients Group

The results in table (3-3) reveal that there were no significant differences in triglyceride (TG) or lipid profile levels between the two groups.

Table (3-3): Lipid profile values in the control group and the type 2 diabetes group.

Variables	**Groups**		**p-value**
	Control(n=50) mean ±SD	Diabetics (n=110) mean ±SD	
Triglyceride	135 ± 22.71	102.78 ± 59.98	0.173 [NS]
Lipid Profile	202.27 ± 8.85	183.77 ± 27.54	0.367 [NS]
Results are presented as mean ± SD, p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant			

3.2.3 Kidney Profile of Healthy Control Group and type2 Diabetic Patients Group

Serum creatinine and blood urea levels differed significantly (table 3–4), with the diabetic group having significantly higher values. Nonetheless, there were no appreciable variations in the blood sodium level between the two groups.

Table(3-4): kidney profile parameters in the control group and type2 DM group.

Variables	**Group**		**p-valaue**
	Control (n=50) mean ± SD	Diabetics (n=110) mean ± SD	
S. sodium (mg/dl)	137.18± 11.87	129.49± 24.65	0.517 [NS]
S. creatinine	0.64± 0.289	1.007± 0.745	0.027 [S]
B. Urea	15.45± 6.87	76.42± 9.48	0.042[S]
Results are presented as mean ± SD, p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant			

3.3 Genetic Analysis

The analysis aimed to evaluate the correlation between the SLC5A2 polymorphisms (rs121918621) and (rs138803748) and the pathogenesis of type 2 diabetes mellitus.

 the SLC5A2 polymorphism (rs121918621) and (rs138803748) as shown in figure (3-1) and figure (3-2).

Figure (3-1): the SLC5A2 polymorphism (rs121918621)

Figure (3-2): the SLC5A2 polymorphism (rs138803748)

Table (3-5): Alleles frequencies of SLC5A2 gene in control and diabetic patients.

Allelels	Groups		p-value
	controls	diabetics	
GG	53 (35.3%)	77 (38.5%)	0.652 [NS]
AG	52 (34.7%)	72 (36%)	
AA	45 (30%)	51 (25.5%)	
The results are shown as percentages and numbers, with [NS] denoting non-significant and p<0.05 indicating a significant difference.			

3.4 Effect of the SLC5A2 Polymorphism (rs121918621), (rs138803748) on the Metabolic Response to empagliflozin.

3.4.1 Effect of the SLC5A2 polymorphism (rs121918621) on the Glycemic Profile of Diabetic Patients in Response to empagliflozin

The results in a table (3-6) showed the level of (FSI, FBG, HbA1c, S. Sodium, Triglyceride, Lipid Profile) were no significant differences. The results also showed the levels of (S. creatinine, B. Urea) were significantly differences.

Table (3-6): chemical parameters in the diabetic patients according to rs121918621.

paramters	***rs121918621***			***P-value***
	AA mean ±SD	**AG mean ± SD**	**GG mean ± SD**	
S. Sodium (mg/dl)	128.86± 37.11	125.43± 41.35	130.55 ± 27.57	**0.064 [NS]**
FBG (mg/dl)	142.92 ± 24.98	146.72 ± 28.82	161.43 ± 26.3	**0.062 [NS]**
HBA1C	6.22 ± 3.25	6.87 ± 3.56	8.09 ± 3.36	**0.054 [NS]**
FSI (mg/dl)	13.03 ± 2.41	11.52 ± 2.89	13.19 ± 1.26	**0.078 [NS]**
Triglyceride	133.53 ± 27.98	126.26 ± 27.47	133.16 ± 32.72	0.279 [NS]
Lipid Profile	182.44 ± 35.66	180.63 ± 38.89	179.46 ± 34.18	**0.068 [NS]**
S. creatinine	0.711 ± 0.133	0.95 ± 0.203	1.42 ± 0.156	**0.014 [S]**
B. Urea	69.82± 10.15	67.31±9.55	75.83± 10.19	**0.042 [S]**
Results are reported as mean ± SD, with p<0.05 regarded substantially different. [S] indicates significant, [NS] indicates non-significant. a; AA vs. AG, b; AA vs. GG c; AG vs. GG				

3.4.2 Effect of the SLC5A2 polymorphism (rs138803748) on the Glycemic Profile of Diabetic Patients in Response to empagliflozin.

Table 3-7 shows that there were no significant variations in the levels of sodium, FBG, and triglyceride. while significant variations in the values of (HBA1C, FSI, Lipid Profile, S. creatinine, and B. Urea).

Table (3-7): chemical parameters in the diabetic patients according to rs138803748.

parameters	**rs138803748**			***P-value***
	AA **mean ±SD**	***AG*** **mean ± SD**	***GG*** **mean ± SD**	
S. Sodium (mg/dl)	120.32± 33.04	121.25± 39.14	124.17 ± 31.27	**0.078 [NS]**
FBG (mg/dl)	137.74 ± 23.76	144.56 ± 27.72	162.36 ± 24.95	**0.057 [NS]**
HBA1C	6.59 ± 2.11	6.18 ± 2.24	7.104 ± 2.53	**0.014 [S]**
FSI (mg/dl)	12.28 ± 3.42	10.63 ± 2.41	14.52 ± 1.98	**0.019 [S]**
Triglyceride	137.94 ± 35.26	128.11 ± 24.37	135.45 ± 39.25	0.174 [NS]
Lipid Profile	185.17 ± 42.19	178.92 ± 41.59	181.54 ± 37.32	**0.036 [S]**
S. creatinine	0.876 ± 0.133	0.93 ± 0.188	1.25 ± 0.128	**0.025 [S]**
B. Urea	75.35± 11.34	68.94±10.83	77.93± 9.29	**0.038 [S]**
Results are reported as mean ± SD, p<0.05 deemed substantially different, [S]=significant, [NS]=non-significant. a; AA vs. AG, b; AA vs. GG c; AG vs. GG				

As shown in Table (3-8), the logistic analysis of rs121918621 in diabetic patients based on glycemic control indicated no significant variations in HbA1c and S. creatinine levels between the mutant allele (AA), heterozygous allele (GA), and wild allele (GG). Similarly, there were no significant variations in the response to empagliflozin across both parameters.

Table (3-8): Regression analysis of rs121918621 with HbA1c and S. creatinine in diabetic patients.

Parametrs	**rs121918621**	**OR (95% CI)**	**p-value**
HbA1c	**GG (66)**	1a	-
	AG (21)	0.091 (0.043-6.605)	0.162 [NS]
	AA (23)	0.446 (0.053-5.221)	**0.098[NS]**
S. creatinine	**GG wild**	1a	**-**
	AG hetero	0.549 (0.048-6.83)	0.53 [NS]
	AA mutant	0.298 (0.093-9.548)	**0.44[NS]**
Odds ratio, confidence interval (CI), and significance level (p<0.05) are used to indicate differences in the results. a : Reference category; confidence interval,			

As shown in Table (3-9), the logistic analysis of rs121918621 in diabetic patients, based on glycemic control, revealed no significant differences in the levels of HbA1c and serum creatinine among the mutant allele (AA), heterozygous allele (GA), and wild allele (GG). Additionally, there were no significant differences observed in the response to empagliflozin for both parameters.

Table (3-9): Regression analysis of *rs138803748* HbA1c and S. creatinine of the diabetic patients.

Parameters	**rs138803748 alleles**	**OR (95% CI)**	**p-value**
HbA1c	**GG**	1a	-
	AG	1.371(0.916-3.853)	0.232 [NS]
	AA	1.125 (0.66-3.749)	0.078[NS]
S. creatinine	**GG**	1a	-
	AG	0.856 (0.275-2.254)	0.73 [NS]
	AA	1.57 (0.264-3.067)	0.167 [NS]
The numbers represent the results; a difference of p<0.05 is deemed significant, while the symbols [S] and [NS] stand for odds ratio and confidence interval, respectively. a; reference category			

The rs121918621 SNP significantly positively correlated with (FBG, HbA1c, FSI, S. creatinine, B. urea), according to the data in table (3–10). and a non-significant relationship with the lipid profile, triglycerides, and sodium (S.).

Table (3-10): The correlation between the chemical parameters of diabetes patients and rs121918621.

rs121918621	**Correlation coefficient(R)**	**P- value**
S. Sodium (mg/dl)	0.367	**0.079 [NS]**
FBG (mg/dl)	0.635	**0.048 [S]**
HBA1C	0.652	**0.033 [S]**
FSI (mg/dl)	0.498	**0.094 [NS]**
Triglyceride	0.187	**0.635 [NS]**
Lipid Profile	0.281	**0.085 [NS]**
S. creatinine	0.794	**0.026 [S]**
B. Urea	0.66	**0.035 [S]**
p<0.05 considered significantly different, [S]= Significant, [NS]= Non significant		

Chapter Four

Discussion

4. Discussion

4.1 Socio-Demographic Data

Diabetes, which is defined by improper metabolism of fat and glucose, is the most common metabolic disease in humans and one of the most important health issues in the world (Navabi, Navabi et al. 2020). The pathophysiological anomalies associated with type 2 diabetes include decreased pancreatic beta-cell insulin output and/or decreased insulin action as a result of insulin resistance. Both the environment and genetics are significant risk factors for type 2 diabetes(Al-Kuraishy, Hamada et al. 2016). Having normal body weight is crucial in the prevention of type 2 diabetes, regardless of genetic predisposition (Schnurr, Jakupović et al. 2020).

Table 3-1 shows a demographic analysis of 110 diabetics and 50 healthy persons (control group). The participants varied in age from 29 to 73 years, with a mean and standard deviation of 41.78 ± 4.99 for the control group and 42.89 ± 4.59 for diabetes patients. There were no statistically significant variations in age between diabetes patients and control subjects. The BMI for the control group was 28.17 ± 3.26, while for patients, it was 33.18 ± 3.32. This outcome indicates a significantly higher BMI in individuals with T2DM compared to the healthy control group, emphasizing the association between obesity and the development of insulin resistance (Cho, Lee et al. 2019, Bilgin, Kurtkulagi et al. 2022) . The study revealed that individuals with obesity and unhealthy lifestyle habits face an increased risk of developing type 2 diabetes, irrespective of their genetic predisposition.

Empagliflozin improves glycemic control, reduces weight, and does not increase the risk of hypoglycemia. It also leads to lower insulin requirements, making it beneficial for obese patients with type 2 diabetes who struggle to achieve adequate control with high multiple dose insulin (MDI) regimens (Chawla and

Chaudhary 2019) Clinical trials have demonstrated that SGLT2 inhibitors, besides lowering HbA1c levels, also contribute to weight loss, lower systolic blood pressure, and decrease serum uric acid. These multifaceted effects suggest the potential for SGLT2 inhibitors to reduce cardiovascular risk in individuals with type 2 diabetes mellitus (T2DM) (Cowie and Fisher 2020).

A family history of type 2 diabetes was found to be a significant factor in our study, whereas there were no notable differences in occupation between the groups under investigation.

Clinical trials revealed a correlation between a family history of diabetes and the response to empagliflozin treatment compared to other antidiabetic drugs(Ku, Lee et al. 2021).

Family history (FH) of diabetes used to be the primary surrogate measure of heritable contribution to diabetes(Ding, Ahmad et al. 2020), Compared with people without a diabetic family member, people who have either one parent or one full sibling with diabetes have approximately a twofold elevated risk of diabetes(Silverman-Retana, Hulman et al. 2020).FH not only reflects heritability, but also reflects potential shared environmental risk factors among relatives(Ye, Niu et al. 2021).

4.2 Assessment of Metabolic Parameters for Control Group and Type2 Diabetes Mellitus Group.

A significant difference in all glycemic induces (FSI, FB G, and HbA1c levels) between the research groups. Diabetes patients had much greater glycemic indices than the healthy group, with the exception of FSI, which was significantly lower in diabetes patients.

Our findings were consistent with Satilmis Bilgin et al (2022) that empagliflozin medication could enhance metabolic parameters in type 2 diabetes individuals(Bilgin, Kurtkulagi et al. 2022) . In another trial, the HbA1c, fasting plasma glucose were all reduced by all SGLT-2 inhibitors, including empagliflozin (Zaccardi, Webb et al. 2016) . A Korean study found that 12 weeks of SGLT-2 inhibitor medication (containing empagliflozin) effectively lowered HbA1c and fasting glucose in the Korean type 2 diabetes population(Hong, Koo et al. 2019).

Although the patients were taking the drug empagliflozin alone, it is possible that if it was combined with another diabetes drug such as metformin or others, we would notice more therapeutic results, and this is confirmed by the study according to Rosenstock *et al.*, when empagliflozin was added to metformin treatment in individuals with type 2 diabetes mellitus, it significantly reduced HbA1c and fasting glucose (Rosenstock, Seman et al. 2013).

Our findings align with those of Takahiro Sawada et al(2019), who observed that SGLT2 inhibitors, including empagliflozin, improved several metabolic indices and reduced plasma triglyceride levels (Sawada, Uzu et al. 2019). Another study conducted by Homa Taheri et al (2023) indicates that in the empagliflozin group, there were non-significant reductions in total cholesterol and triglyceride levels compared to the placebo group (Taheri, Chiti et al. 2023).

According to a previous study that found substantial associations between HbA1c and lipid profiles, diabetic individuals with poor glycemic control had significantly higher levels of TG and lower levels of HDL, suggesting the significance of appropriate diabetes care in regulating dyslipidemia(Nnakenyi, Nnakenyi et al. 2022).

Patients with diabetes frequently have dyslipidemia, hyperlipidemia, and poor glucose control. The combined risk of macro and microvascular problems is increased by hyperglycemia and dyslipidemia, despite the fact that both conditions are substantial independent risk factors for vascular complications. The etiology of vascular problems in people with type 2 diabetes may involve dyslipidemia, which is characterized by both quantitative and qualitative abnormalities of lipoproteins(Hasheminasabgorji and Jha 2021).

We can clearly and more accurately determine that triglycerides and cholesterol are factors that can be affected by the occurrence of diabetes and the use of empglifizone medication. If the results had been taken from a large number of samples in our study and on a large scale... taking into account other factors such as LDL and HDL.

Hyperuricemia (HUA) is a metabolic disease characterized by elevated serum uric acid (SUA). Empagliflozin, a kind of sodium-glucose cotransporter 2 inhibitors, has recently emerged as a new antidiabetic agent by facilitating glucose excretion in urine(Lu, Chang et al. 2020).

Table (3-4) showed significant differences in B. Urea and S. Creatinine The group with diabetes had a considerably higher creatinine level. While the Serum sodium level showed non-significant differences between the two groups.

As reported in previous study that in patients with T2DM have possibility to cardiovascular risk , the SGLT inhibitor reduce HbA1c and blood pressure and serum uric acid and body weight so decrease the accident of cardiovascular disease (Cowie and Fisher 2020).

For lowering the need for kidney replacement therapy by over 40%, SGLT2 inhibitors may double of serum creatinine levels. The molecular mechanisms behind these beneficial effects of SGLT2 inhibitors extend beyond their glucose-lowering effects. The emerging studies are trying to explain these mechanisms at the genetic, epigenetic levels(Klen and Dolžan 2023). And this is consistent with our findings, which show that the successful use of Empagliflozin medication appears to Our data show a considerable decrease in hyperuricemia among diabetes individuals. A research done by Hu et al., combining randomized controlled trials with individuals who had type 2 diabetes in Asia, revealed that SGLT-2 inhibitors, particularly Empagliflozin, significantly decreased serum uric acid (SUA) levels (Hu, Yang et al. 2022). Other accessible trials have demonstrated that SGLT2 inhibitors, including canagliflozin, dapagliflozin, and empagliflozin, significantly lower serum uric acid (SUA) levels compared to placebo (Xin, Guo et al. 2019).

Furthermore, Ferreira et al. conducted investigations on diabetic individuals and discovered that empagliflozin medication resulted in a decrease in serum uric acid (SUA) levels compared to placebo(Ferreira, Inzucchi et al. 2022).

The indicated results were similar with the findings of the current investigation, which showed that empagliflozin in dosages of 10 mg is beneficial in treating hyperuricemia in individuals with type 2 diabetic mellitus (T2DM). These data confirm empagliflozin's efficacy in decreasing serum uric acid (SUA) levels in T2DM patients.

Through the results of our study and previous research, we can say that the decrease in serum creatinine and uric acid in response to the drug empagliflozin in diabetic patients is not limited to people with diabetes only, but also to people with diabetes mellitus, gout, and diabetes associated with kidney disease. These clinically important findings expand the clinical utility of empagliflozin and The mechanism behind this apparent effect deserves further investigation(Ferreira, Inzucchi et al. 2022).

4.3 Genetic Analysis

Through our study, which included the treatment of empagliflozin for diabetics, which is a drug belonging to the group of sodium-glucose transporters 2, it is a drug that works to inhibit sodium and glucose transporters in the kidneys, and an attempt to understand and study the effect of the gene for the transporter on the drug response.

The current study investigated in a group of the Iraq population the response of T2DM with rs121918621, rs138803748 *SLC5A2* genotypes to empagliflozin treatment.

4.4. Influence of SLC5A2 polymorphism on empagliflozin response in patient with type 2 DM.

A 243 genetic loci have been discovered by researchers in the past ten years while they look into the underlying genetic etiology of type 2 diabetes mellitus (T2DM). The identified genes, however, only contribute around 20% of the genetic risk (heritability) associated with type 2 diabetes, similar to many other polygenic illnesses (Sun, Kristiansen et al. 2019). This study investigated the association

between the pathogenicity of type 2 diabetes mellitus and the SLC5A2 polymorphisms (rs121918621 and rs138803748). The frequencies ofSLC5A2 alleles were not significantly different between the control andT2DM groups, according to the data in table (3-5). This suggests that the pathogenicity of type 2 diabetes and the SLC5A2 SNPs (rs121918621 and rs138803748) are unrelated.

In tables (3-6), the SNP rs121918621 *SLC5A2* genotype that showed levels of (FSI, FBG, HbA1c, S. Sodium, Triglyceride, and Lipid Profile) showed no significant differences. While the levels of S. creatinine and B. urea showed significant differences. Although each *SLC5A2* genotype has a different genetic influence than the others, we found the (rs121918621, rs138803748) *SLC5A2* genotypes to have the same significant response to (S. creatinine, B. Urea). This is due to the improved effect of the empagliflozin drug on (S. creatinine, B. Urea).

As we compare with the result of another SNP rs138803748 *SLC5A2* genotypes table (3-7) that show all chemical parameters give a significant response except (FBG, S. Sodium, Triglyceride) were no significant differences. the data in the table (3-8) SNP rs121918621for logistic regression analysis, and table (3-9) for SNP rs138803748 *SLC5A2* genotypes. The results indicated there is no significant association between different alleles and the pathogenesis of type 2 diabetes mellitus, as well as the response to empagliflozin.

4.5 Estimation of risk in SLC5A2 gene (rs121918621, rs138803748) in relation to empagliflozin response diabetic patient

The results in tables (3-8) and (3-9) demonstrated that the wild allele GG had a higher decrease in HbA1c and S. creatinine levels than the mutant allele AA for each SNP. These data indicate that individuals with mutant alleles had considerably less responses to empagliflozin, potentially due to diminished or eliminated SGLT2 function.

Table 3-10 shows the findings of the association between the chemical parameters and the rs121918621 SNP of SLC5A2, which demonstrated a strong positive correlation with FBG, HbA1c, S. creatinine, and B. urea levels. This suggests that these measures in diabetes individuals were enhanced as a result of the SLC5A2 SNP, indicating a reduction in empagliflozin activity due to the polymorphism. In comparison to table (3-11), there is a substantial positive association with rs138803748 HbA1c ($p<0.05$) but no significant correlation with other measures. This demonstrated that HbA1c was the most heavily influenced by genetic variation induced by two SNPs.

Our study found that the SLC5A2 rs121918621, rs138803748 polymorphism substantially elevated rapid serum insulin, blood urea, and serum creatinine levels in T2D patients ($P<0.05$). Furthermore, as shown in table (3-6), carriers of the polymorphic SLC5A2 rs121918621 GG natural allele exhibited greater mean and standard deviation of S. creatinine and B. urea than mutant alleles AA and AG. While the SLC5A2 rs121918621 polymorphism had no significant impact on fasting blood glucose, HbA1c, and fasting serum insulin levels in T2D patients.

Zimdahl H. et al. (2017) stated that there was no significant connection between the tested SNP and HBC1A, fasting blood glucose, or fasting blood insulin in a

cross-sectional investigation. The results of our study were consistent with their findings. Additionally, there was no clinically significant impact on the response of type 2 diabetes patients to the SGLT2 inhibitor empagliflozin, which was a finding that contradicted our findings(Zimdahl, Haupt et al. 2017).This disagreement may be attributed to dosage form of drug and use of combination drug with empagliflozin that may be impact on pharmacological effect as well as number of sample size.

A prior study that used a single dosage of the SGLT2 inhibitor empagliflozin in a pharmacogenetic analysis found no substantial and clinically meaningful effects of SLC5A2 SNPs prevalent in HbA1c, fasting glucose, body mass index, or hypertension(Zimdahl, Haupt et al. 2017).

According to a meta-analysis by Zhao et al. (2018), empagliflozin decreases fasting plasma glucose, uric acid, and HbA1c.These results suggest beneficial effects of empagliflozin on renal and glycemic indices (Zhao, Liu et al. 2019).

4.6 Conclusions

1- SLC5A2 polymorphisms among genes influencing variation in response to empagliflozin in patients with type 2 diabetes This indicates the importance of SLC5A2 polymorphisms in variation in response to empagliflozin in patients with 2DM .

2. Individuals with heterozygous and wild-type SLC5A2 SNP alleles had different responses to empagliflozin treatment. On the other hand, patients with mutated alleles of the SLC5A2 SNP showed no significant response to empagliflozin treatment.

3. No association between SLC5A2 SNP polymorphism and onset of type 2 diabetes.

4- Investigating pharmacogenetic mechanisms as performed in this study holds the promise of providing new insights into factors affecting metabolism. In addition, potential therapeutic targets may be identified, opening the way for precise intervention in type 2 diabetes mellitus (T2DM) patients.

4.7 Recommendations and Suggestions

1- Our recommendation is to examine genetic tests that can predict an individual's response to empagliflozin therapy and this process can lead to the development of personalized medicines with properties that are effective and safe.

2. Further studies are needed to evaluate the effect of other genetic variants associated with response to empagliflozin.

3.study of antidiabetic effect of empagliflozin on T2DM.

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الخلاصة

المقدمة: داء السكري من النوع 2 هو مرض متعدد الجينات غير متجانس للغاية. وهو مرض معقد متخصص في ارتفاع السكر في الدم. T2DM هناك اختلافات معينة في النمط الظاهري لمرضى T2DM بسبب التباين الوراثي والاختلافات في نمط الحياة والتعرضات البيئية الأخر مثبطات ناقل الجلوكوز الصوديوم 2 (SGLT2) هي عوامل جديدة لخفض السكر في الدم تزيد من إفراز الجلوكوز في البول عن طريق تثبيط إعادة امتصاص الجلوكوز في الأنابيب القريبة من الكلى، وهو ما يعتبر مفهومًا جديدًا في علاج مرض السكري من النوع 2.

إمباغليفلوزين هو دواء جديد نسبيًا يعمل على تحسين إدارة نسبة السكر في الدم عن طريق زيادة إفراز الجلوكوز في البول وتثبيط الناقل المشترك للصوديوم والجلوكوز2 (SGLT2).

هدف الدراسة: ـ دراسة العلاقة بين تعدد أشكال النوكليوتيدات المفردة (SNPs) في الجين SLC5A2 المشفر SGLT2 (rs121918621) و ((rs138803748) مع تأثير الإمباجليفلوزين في مرضى السكري.

طرق العمل؛ شمل المشاركون في الدراسة 50 فرداً أصحاء و110 مرضى السكري الذين تم اختيارهم خلال زياراتهم للعيادات الخارجية. خضع كل شخص لتقييم نسبة السكر في الدم، ودراسة وراثية لتعدد الأشكال (rs121918621) SLC5A2 و((rs138803748) .تلقى كل مريض جرعة يومية قدرها 10 ملغ من إمباجليفلوزين كعلاج وحيد.

النتيجة: كشفت نتائج الدراسة عن اختلافات كبيرة في كل من مؤشرات نسبة السكر في الدم ومؤشر كتلة الجسم بين مجموعات الدراسة، حيث كانت مؤشرات نسبة السكر في الدم لدى مرضى السكري أعلى بشكل ملحوظ من تلك الموجودة في العينة السليمة. أظهر التوزيع الوراثي (SLC5A2(rs121918621 و((rs138803748) وجود فروق معنوية في مستوى ((FSI, HbA1cبينما لم يكن مستوى ((FBGفروقاً معنوية.

الاستنتاج: من المحتمل ان يكون تعدد الأشكال (rs121918621) SLC5A2 و ((rs138803748أحد المتغيرات الوراثية المسببة للاختلاف في طريقة استجابة مرضى السكري العراقيين للإمباجليفلوزين

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء

كلية الصيدلة

دراسة تأثير تعدد الأشكال في جين SLC5A2 على الاستجابة لعلاج empagliflozin لدى مرضى السكري من النوع الثاني

رسالة مقدمة الى كلية الصيدلة - جامعة كربلاء

كجزء من متطلبات نيل درجة الماجستير في علم الأدوية والسموم

من قبل

بنين هادي سعيد

(بكالوريوس صيدلة – جامعة الكوفة 2008)

بأشراف

الأستاذ المساعد الدكتور

محمد إبراهيم رسول

2024 ميلادي

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