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EDITORIAL

Potential therapeutic targets for the prevention of diabetic nephropathy: Glycyrrhetinic acid

Lu Cai, Michael Horowitz, Md Shahidul Islam

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Abstract

Uncontrolled hyperglycemia or poorly managed disease increases the propensity for a number of diabetes-related complications targeting major organs including the heart, eyes, and kidney. Although the mechanisms by which diabetes induces cardiovascular diseases include oxidative stress and inflammation, when insulin resistance remains the key to the pathogenesis, as implicated in the two reviews in this issue. This editorial mainly comments on the potential preventive application of glycyrrhetinic acid (or 18β-GA) in relation to diabetic nephropathy. The therapeutic or preventive effects of 18β-GA, as a hydrolytic product of glycy-rrhizic acid that is a component of licorice, have been appreciated in other disorders, but have received much less attention in relation to diabetic complications. A study in this issue has identified 18β-GA as a therapeutic for preventing diabetic nephropathy and provides evidence to support efficacy in cultured human renal tubule cells *in vitro*. Although it represents a pilot study, the observations support a new therapeutic approach that warrants further ex-ploration.

Key Words: Insulin resistance; Diabetic cardiomyopathy; Diabetic nephropathy; Glycyrrhetinic acid; Licorice; Chinese herbal remedy

Core Tip: Uncontrolled hyperglycemia or poorly managed disease increases the propensity for a number of diabetes-related complications targeting major organs including the heart, eyes, and kidney. Although the mechanisms by which diabetes induces cardiovascular diseases include oxidative stress and inflammation, when insulin resistance remains the key to the pathogenesis, as implicated in the two reviews in this issue. This editorial mainly comments on the potential preventive application of glycyrrhetinic acid (or 18β-GA) in relation to diabetic nephropathy (DN). The therapeutic or preventive effects of 18β-GA, as a hydrolytic product of glycyrrhizic acid that is a component of licorice, have been appreciated in other disorders, but have received much less attention in relation to diabetic complications. A study in this issue has identified 18β-GA as a therapeutic for preventing DN and provides evidence to support efficacy in cultured human renal tubule cells *in vitro*. Although it represents a pilot study, the observations support a new therapeutic approach that warrants further exploration.

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INTRODUCTION

Diabetes is well known as a chronic, metabolic disease that over time often leads to serious damage to the heart, blood vessels, eyes, kidneys and nerves. More than 95% of people with diabetes have type 2 diabetes mellitus (T2DM). T2DM occurs when the body becomes resistant to insulin or doesn't make sufficient insulin due to partial pancreatic b-cell damage. T2DM usually occurs in adults but a number of recent studies have demonstrated its disturbing increasing prevalence in children and adolescents[1,2]. Uncontrolled hyperglycemia or poorly managed diabetes leads to a number of diabetes-related complications targeting major organs, including heart, kidney, brain and eyes. Although these potentially life-threatening complications can be reduced or delayed by following healthy lifestyle, awareness of warning signs, regular visits to a health care provider, and effective therapeutic interventions are urgently needed, as discussed in two reviews in this or recent issue[3,4]. The mechanisms by which diabetes induces cardiovascular diseases include oxidative stress and inflammation[1-4]. However, insulin resistance remains key to the pathogenesis, underpinning the increases in oxidative stress and inflammation, particularly in individuals with T2DM[1-4]. Insulin resistance is often accompanied by hyperlipidemia, therefore, atorvastatin as one of the statins, HMG-CoA reductase inhibitors, are used widely as a class of lipid-lowering medications. Song *et al*[5] reported their efficacy in preventing diabetic cardiomyopathy in db/db T2DM mice, which may also be associated with anti-oxidative and anti-inflammatory effects through modulating the polarization of macrophages. This information provides an additional rationale for statins in the management of diabetic complications. However, these drugs may also exhibit adverse effects on other organs. Accordingly, the importance of natural compounds for the management of diabetic complications cannot be underestimated due to their minimal adverse effects. These natural compounds include, but not limited to, polyphenols, flavonoids, phenolic acids and zinc that have been shown to have substantial beneficial effects in the management of hyperglycemia, diabetes and its associated complications[6]. In line of this notion, a study by Meng *et al*[7] in a recent issue proposed the potential preventive effect of glycyrrhetinic acid (also called 18β-Glycyrrhetinic acid, 18β-GA) for the management of diabetic nephropathy (DN).

18β-GA, as a hydrolytic product of glycyrrhizic acid, is a component of licorice. Licorice (sometimes spelled liquorice) has been used as an herbal remedy and sweetening agent across cultures for centuries. Chinese licorice (or liquorice) root is the rhizome (the underground stem) of the plant Glycyrrhiza glabra, which is native to Asia, Turkey, and Greece. Glycyrrhizic acid is structurally composed of two molecules of glucuronic acid and 18β-GA. Glycyrrhizic acid is metabolized by gut bacteria to 18β-GA[8-10]. Therefore, 18β-GA is an *in vivo* metabolic component of glycyrrhizic acid. It is considered widely as one of the main active substances of licorice. Although a number of recent studies have focused on the biological activities of 18β-GA, this has related primarily to its anti-inflammatory, immunoregulatory, anti-tumor, anti-injury, and antioxidative properties. A number of comprehensive reviews have summarized the protective effects of licorice-derived 18β-GA against liver injury[8] and its potential immunomodulatory and anti-inflammatory properties[9], and efficacy in cancer therapy[10], as summarized in Figure 1. However, less work has been done in relation to its potential in the management of diabetic complications.

Although many studies have shown the beneficial effects of 18β-GA on other conditions, its application, and associated mechanisms for the preventive and therapeutic effects on diabetic complications are poorly understood[11,12]. The study by Meng *et al*[7] in this issue, explored the therapeutic targets and molecular mechanisms of 18β-GA against DN based on network pharmacology and molecular docking, and found that 18β-GA has a therapeutic effect on DN with a potential 186 targets. Molecular docking studies demonstrated strong binding of 18β-GA to mitogen-activated protein kinase (MAPK)-1, SRC, PIK3R1, HSP90AA1, CASPASE9, HARS, KRAS, and MAPK14. It was revealed that 18β-GA inhibits HK-2 cell viability, induces cell cycle arrest at the G2/M phase, and reduces apoptosis with 18β-GA in a dose-dependent manner after the treatment of an immortalized proximal tubule epithelial cell line from normal adult human kidney cells or HK-2 cells with high level of glucose with and without 18β-GA. Further analysis showed that 18β-GA differentially up-regulated key insulin signaling pathway members including PI3K, AKT and GSK3. These innovative and important

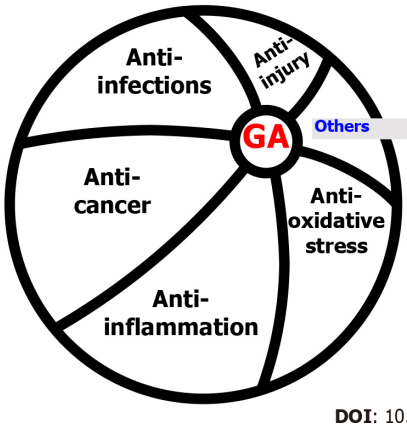

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Figure 1 Summary of the beneficial effects of glycyrrhetinic acid family members. The left panel summarizes the well-known beneficial effects based on several reviews cited in this editorial publication[8-10]. The right panel of this figure as a small part of total research on provides the limited publications that showed the certain protective effects on metabolic syndrome, hyperglycemia, and potential diabetic complications. GA: Glycyrrhetinic acid.

observations have provided the evidence to support the concept of therapeutic efficacy as published previously[11,12].

CONCLUSION

In conclusion, as a main component of licorice, 18β-GA-mediated beneficial effects in several pathogenic conditions have been widely appreciated; however, its application to the management of diabetes and diabetic complications remains elusive. Although the outcomes of this pilot study have raised many questions that should be further addressed, as the authors propose, it also provides a new direction in relation to its potential clinical application for the prevention and management of DN, which is likely to be safe, inexpensive and with no or lower adverse effects.

FOOTNOTES

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REVIEW

Analysis of the management and therapeutic performance of diabetes mellitus employing special target

Hong-Yan Sun, Xiao-Yan Lin

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Abstract

Diabetes mellitus (DM) is a chronic metabolic condition characterized predominantly by hyperglycemia. The most common causes contributing to the pathophysiology of diabetes are insufficient insulin secretion, resistance to insulin's tissue-acting effects, or a combination of both. Over the last 30 years, the global prevalence of diabetes increased from 4% to 6.4%. If no better treatment or cure is found, this amount might climb to 430 million in the coming years. The major fact-ors of the disease's deterioration include age, obesity, and a sedentary lifestyle. Finding new therapies to manage diabetes safely and effectively without jeopardizing patient compliance has always been essential. Among the medications available to manage DM on this journey are glucagon-like peptide-1 agonists, thiazolidinediones, sulphonyl urease, glinides, biguanides, and insulin-targeting receptors discovered more than 10 years ago. Despite the extensive preliminary studies, a few clinical observations suggest this process is still in its early stages. The present review focuses on targets that contribute to insulin regulation and may be employed as targets in treating diabetes since they may be more efficient and secure than current and traditional treatments.

Key Words: Diabetes mellitus; Hyperglycemia; Therapeutic performance; Management; Special target; Literature review

Core Tip: Diabetes mellitus (DM) is a chronic metabolic condition characterized by hyperglycemia. Major contributing factors are insufficient insulin secretion, insulin resistance, or both. Global diabetes prevalence has risen from 4% to 6.4% in the past 30 years and may reach 430 million in the future. Age, obesity, and a sedentary lifestyle exacerbate the disease. Developing safe and effective therapies is crucial. Medications like glucagon-like peptide-1 agonists, thiazolidinediones, and others have been available for over a decade. However, clinical observations suggest ongoing research. This review focuses on insulin regulation targets for potentially more efficient and secure diabetes treatments.

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INTRODUCTION

Mellitus, short for "sugar disease", pertains to the endocrine conditions known as diabetes. Diabetes mellitus (DM), one of the most common conditions, is presently the seventh leading cause of death, with 5.2 million deaths worldwide[1]. Diabetes that is either untreated or poorly managed is thought to be the cause of 1.5 million deaths annually globally. From 108 million cases (4.7%) in 1980 to 425 million patients (8.5%) in 2017, it is expected that 629 million people will have diabetes by 2045. The cost of treating diabetes worldwide is predicted to be 760 billion USD per year, with costs being the same for men and women[2]. Both inadequate insulin production by the pancreas or elevated glycosylated hemoglobin and improper insulin response by bodily cells contribute to the development of DM[3]. In addition, the development of diabetes can be influenced by a wide variety of factors, including a lack of physical activity, excessive consumption of food and beverages, obesity, stress, and industrialization. Environmental and genetic factors are the primary causes of diabetes[4]. Diabetes can cause many health problems if not treated, such as chronic hyperglycemia, which can cause long-term damage to the blood vessels, heart, eyes, nerves, kidneys, and other organs[5].

Diabetes is classified into three types: Type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes (GD), and other variants. T1D affects approximately 5%-10% of individuals diagnosed with it, usually young children and teenagers [6]. A complete lack of insulin production brings on type 1 DM (T1DM). Type 2 DM (T2DM), which is much more common, is caused by inadequate corrective insulin secretory response and resistance to insulin action[7]. 90%-95% of people with diabetes have T2DM, the most common type, which continues to increase worldwide[8]. In the 20 years following delivery, GD increases the chance of T2D by 35%-60%. One in every six live births is complicated by GD, which occurs in the second or third trimester of pregnancy[8]. Drug-induced diabetes, pancreatic illness, and monogenic diabetes are additional types. Type 1.5 diabetes, also known as latent autoimmune diabetes in adults (LADA), is like T2DM, which occurs gradually throughout development. LADA is an autoimmune disease that cannot be treated by modifying an individual's diet or lifestyle[9].

The proportion of the aging population is increasing, and this trend is explained by urbanization, socioeconomic growth, highly processed diets, and a decline in physical exercise. Untreated diabetes typically causes unintentional weight loss, increased excretion, increased thirst, and increased appetite[10]. T1DM symptoms can appear suddenly, whereas T2DM symptoms typically appear much more gradually and may even be nonexistent. About half of the people do not realize they have T2D because there are few symptoms or signs in the early stages of the disease. As a result, symptoms go undetected and lead to complications associated with diabetes[11]. Glycated hemoglobin (HbA1C), fasting plasma glucose level (126 mg/dL), and plasma glucose (200 mg/dL) tests are used to identify DM. Nowadays, there is no validated prophylactic method for T1DM. By maintaining a healthy body weight, getting exercise, and adhering to a wholesome diet, T2DM can be prevented. Higher levels of physical activity (> 90 min/d) reduce diabetes risk by 28%[11].

Diabetes management aims to keep blood sugar levels near normal without lowering them. This is usually accomplished by making dietary changes, exercising, losing weight, and taking the appropriate medicines. Restoring normal carbohydrate metabolism is the primary aim of DM management and control[12]. Insulin replacement therapy is needed for people with total insulin deficiency. Contrarily, nutritional changes and exercise can be used to treat insulin resistance. Preventing or treating the numerous complications that can arise from the illness and its treatment are other objectives of diabetes management[12]. This review aims to analyze the management and therapeutic performance of DM using specific targets. Our current comprehensive study has identified several potential targets with promising leads that, if further explored, may result in developing the next wave of anti-diabetic medications.

DM PATHOPHYSIOLOGY

Several hormones cooperate to maintain an appropriate amount of glucose in the body. However, two hormones, insulin and glucagon, dominate in regulating glucose homeostasis. When the level of glucose increases, cells in the pancreatic islets of Langerhans produce insulin. Insulin lowers blood sugar levels by preventing the liver's synthesis of glucose through glycogenolysis and gluconeogenesis[13] or by boosting the liver, muscle, and fat tissues' glucose uptake, except for soft muscle, where insulin functions *via* insulin-like growth factor-1. Therefore, all types of DM are caused by insulin

deficiency or receptor insensitivity. Insulin has the following effects: Decreasing gluconeogenesis and inhibiting gluconeogenesis, promoting glucose transport into adipose and muscle cells, and raising glycogen storage[14].

Fewer beta cells produce insulin when glucose levels are low, and less glycogen is converted into glucose. The pancreatic cells secrete glucagon. By accelerating liver functions like glycogenolysis and gluconeogenesis, glucagon enhances the effects of insulin. The cells that require glucose are unable to absorb it, and it is not stored correctly in the liver and muscles if there is not enough available insulin, if cells are resistant to the effects of insulin (insulin resistance), or if the insulin itself is defective[15]. Consistently high blood glucose levels, impaired protein synthesis, and metabolic anomalies like metabolic acidosis are the results of severe insulin deficiency. Maintaining a high blood glucose level causes the kidneys to reach their reabsorption limit, resulting in the excretion of excess glucose through urine (glycosuria). This leads to polyuria and fluid loss because the osmotic pressure of the urine goes up, and the kidneys take in less water[16]. Dehydration and increased thirst (polydipsia) result from the body osmotically replacing blood volume with water from other sources, such as cells and different bodily compartments.

Additionally, low glucose levels in the blood increase hunger, leading to overeating (polyphagia)[17]. Cortisol and catecholamines also raise plasma glucose levels in addition to glucagon. Glucose-dependent insulinotropic polypeptide, glucagon-like peptide-1 (GLP-1), and amylin are additional hormones that help to maintain an average blood glucose level [glucose-dependent insulinotropic polypeptide (GIP)][18].

Along with insulin, amylin is released. It lessens stomach emptying, improving glucose absorption following a meal. Incretins or peptides produced from the gut include GLP and GIP. These incretins help the pancreatic beta cells produce and secrete insulin[19]. Neither the intestine nor cells in need of energy can easily absorb glucose. Therefore, glucose transporters are responsible for delivering glucose to the cells. Sodium-glucose co-transporter (SGLT) and facilitative glucose transporter are two examples of the two kinds of glucose transporters, which are a family of membrane-bound glycoproteins (GLUT)[20]. The interplay of genetic and environmental factors largely determines T2D. The risks increase with increasing levels of overweight or obesity. Hormonal changes that arise during pregnancy are the cause of GD. Hormones produced by the placenta lessen the sensitivity of cells to the impacts of insulin. DM can result from genetic mutations like a single gene mutation that can produce monogenic diabetes[21].

The most common forms of monogenic diabetes or maturity-onset diabetes of the young are diabetes at birth and diabetes that develops in early adults. Thick mucus is produced by cystic fibrosis, which prevents the pancreas from producing enough insulin, leading to pancreatic scarring. The body stores an excessive amount of iron due to hemochromatosis. Iron can accumulate in the body and harm other organs, including the pancreas, if the condition is not treated [20]. High hormone production levels in the body are a symptom of some hormonal illnesses, which can lead to insulin resistance and diabetes in some people. Excessive levels of cortisol, also known as the stress hormone, cause Cushing's syndrome[21]. Too much growth hormone causes acromegaly[22,23]. When the thyroid gland generates too much thyroid hormone, hyperthyroidism develops. Diabetes is caused by pancreatic damage or removal, including pancreatitis, pancreatic cancer, and trauma. These conditions have the potential to cause harm to β-cells or decrease their ability to produce insulin. Diabetes develops if the damaged pancreas is removed due to β-cell loss[24] (Figure 1).

MANAGEMENT OF DM

Diabetes management aims to boost output and quality of life for people with diabetes by: (1) Early detection; (2) Long-term and short-term morbidity prevention; (3) Early death prevention are all examples of early diagnosis[25]; (4) Supporting diabetes patients' freedom and self-care habits; (5) Reduction of the personal, family, and societal burden of diabetes; and (6) Achieving these objectives depends on the facilities and diabetes health care team being successfully established. This involves educating those with diabetes and healthcare professionals[26].

Blood sugar level

A glucose meter is used to test blood sugar levels, and the results are displayed either in mg/dL or mmol/L of blood. A healthy individual's average fasting glucose level is 4.5 mmol/L (81 mg/dL), ranging from 65 to 98 mg/dL at its lowest and highest points, respectively[27]. The most effective method to manage diabetes is for each patient to keep track of their blood glucose levels and how exercise and food affect them. Patients can improve their diabetes management by changing their habits[28].

Hypo and hyperglycemia

A hypoglycemic episode is a glucose level of 3.8 mmol/L. 55% of cases of severe hypoglycemia occur during sleep in T1D that is well-controlled. 6% of fatalities in people with diabetes under 40 are attributed to nocturnal hypoglycemia (dead-in-bed syndrome)[29]. According to the National Institute of Health data, hypoglycemia accounts for 2% to 4% of all diabetic deaths. After intensive glucose control, 21% of hypoglycemia incidents in children and adolescents were unexplained. In addition to being fatal, hypoglycemia can also cause cerebral damage during severe episodes. Although glucose is typically linked to diabetic nerve disease, hypoglycemia can also start or exacerbate neuropathy in people with diabetes who are actively trying to lower their hyperglycemia[30]. It is essential to carefully monitor levels above 230-270 mg/dL, regarded as high and should be brought down rather than allowed to stay high. Hyperglycemia is the term for high blood sugar levels, which is harder to spot than hypoglycemia and typically develops over days instead of hours or minutes. If unattended, this may cause a diabetic coma and mortality[31].

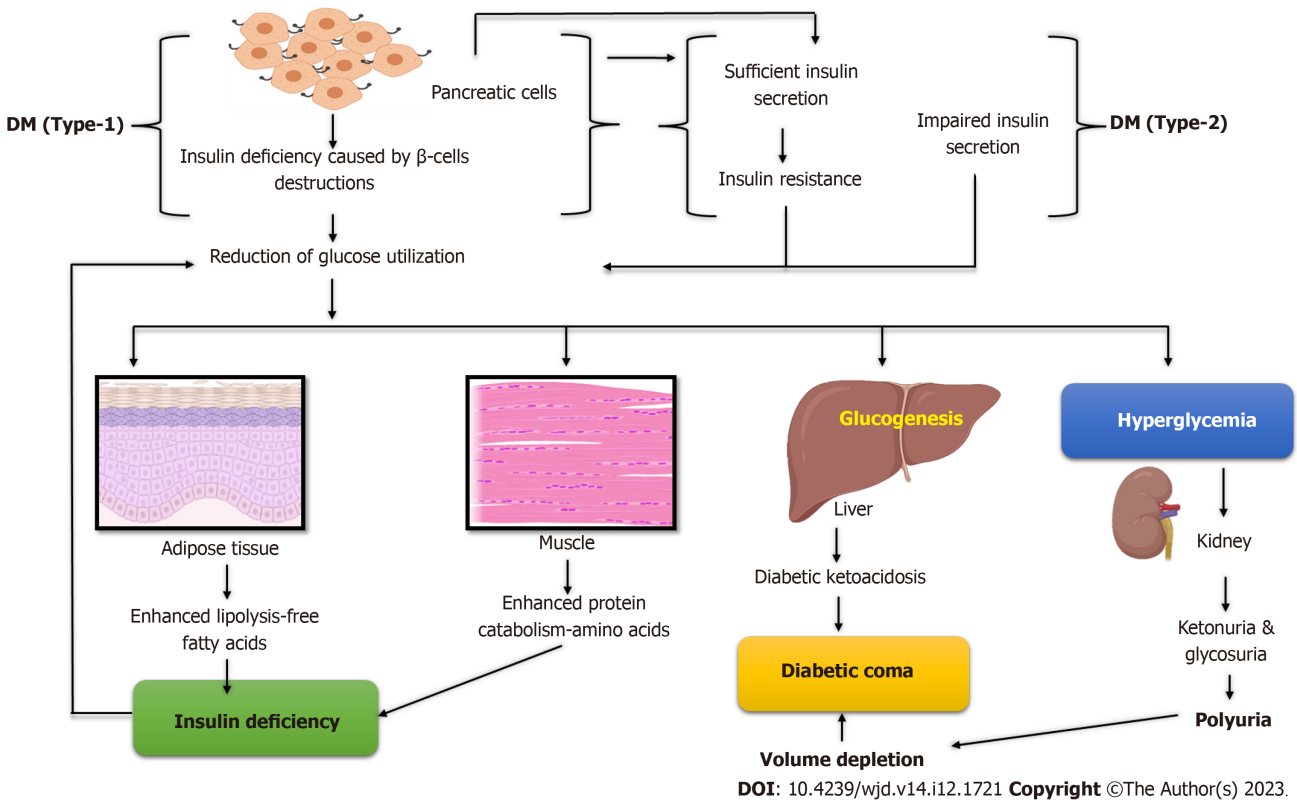

Figure 1 Illustration of the pathophysiology of diabetes mellitus (type 1 and type 2). DM: Diabetes mellitus.

Glycemic control

In medicine, the word "GC" refers to the typical blood sugar levels of a person with DM. Numerous pieces of evidence indicate that years of hyperglycemia cause multiple serious issues of diabetes, especially complications of microvascular origin. Effective glycemic control (GC), in the sense of a "target" for treatment, has become a crucial aim of diabetes care despite recent research suggesting that genetic factors may be accountable for the complications of diabetes[32]. T1D is brought on by the autoimmune condition that first rendered the pancreas incapable of making insulin. Because blood sugar levels vary throughout the day and glucose records are unreliable indicators of these changes, the quantity of HbA1C is a substitute indicator of long-term GC in research studies and clinical therapy for people with diabetes[33].

The hemoglobin A1c test, or HbA1C, measures the average glucose levels over the two to three months prior. By the most popular measures, HbA1C is typically 4%-6% in non-diabetic individuals with average glucose metabolism Blood glucose and HbA1C levels of 11-28 mmol/L and 9%-15% or higher, respectively, over months and years before severe complications develop, are indicative of poor GC[34]. There has been no difference in all-cause mortality, nonfatal stroke, or limb amputation in extensive studies comparing the impact of strict GC to conventional or more relaxed GC in T2D. Still, there has been a 15% decrease in the risk of nonfatal coronary artery disease[35]. Despite being linked to a 2.4-fold higher risk of hypoglycemia, strict glucose control is also related to a lower risk of retinopathy and nephropathy and a lower incidence of peripheral neuropathy[36].

Personal glucose monitoring

Regular home glucose meter use by patients, especially those with T1D, may improve management and outcomes for both type 1 and 2 diabetes. Keeping tabs on one's glucose levels is time-consuming and labor-intensive, not to mention costly. Monitoring blood glucose levels helps keep the illness under control and lessen the likelihood of serious complications later on[37]. There are many different kinds of blood monitoring devices, and each one works for every patient. For those with T1D, self-testing is crucial because insulin therapy can result in low blood sugar (hypoglycemia), and home testing allows you to adjust the dosage each time you administer insulin. A new study suggests that self-monitoring does not improve blood glucose control or quality of life, even though its efficacy in T2D has been more controversial. Despite home blood glucose monitoring, type 2 patients are considered to have poor long-term management[38].

Continuous glucose monitoring (CGM) technology has improved to provide data regarding the pace and pattern of glucose changes in people with diabetes. The accuracy of these devices is improving with each new advancement, although self-monitoring blood glucose calibration remains necessary and is not designed for correction boluses[39]. The CGM and Libre Sensor are used in the Libre Blood Sugar Diet Program, and by collecting all data *via* smartphone and smartwatch, experts can evaluate it in real-time, round-the-clock, every day of the week. As a result, certain foods can be determined to raise blood sugar levels, while others can be identified as being healthy and not doing so. Sugar is absorbed differentially by each individual, so testing is necessary[40].

HbA1c test

The measurement of blood HbA1c levels is a valuable test typically performed in a laboratory. This is the proportion of HbA1C to overall hemoglobin. The percentage of these molecules increases as plasma glucose levels remain elevated. This test, once thought to assess the average level of diabetic control over about three months, has been suggested to emphasize the most recent 2 to 4 wk[41]. HbA1c levels range from 4.0 to 6.0 in people who don't have diabetes. People with diabetes whose HbA1c levels stay < 6.5% are said to have reasonable GC[37]. If diet or treatment adjustments have been made within the last six weeks, or if there is a hemoglobinopathy or a disruption in red cell aging, the HbA1c test is inappropriate. The alternative Fructosamine test shows standard control over the previous two to three weeks[42].

Use of digital tools

People with T2DM can lower their blood sugar levels by sharing their electronic health data with them. It is a method of assisting individuals in understanding their health conditions and actively participating in their administration. About 100000 health-related apps are available on Google Play and the App Store, and the most general category is diabetes applications[43]. Routine self-management activities such as taking medication and insulin, monitoring blood sugar levels, adhering to a diet, and participating in physical exercise present significant challenges. Nevertheless, despite the many applications available, only a relatively small percentage of patients use them[44].

Furthermore, a 2016 study of 65 Android diabetes apps discovered that confidential information, such as insulin and blood sugar levels, "was routinely collected and shared with third parties". One study investigates how a T2D Android mobile application can integrate supporting hardware such as an exercise bike, a treadmill, a heart-rate sensor, a wearable band, and a glucometer. This mobile program includes drugs, food consumption, exercise, and sleep tracking. Adesina *et al*[45] examine the effectiveness and applicability of digital tools designed to assist women with GD dietary self-management.

Foot examination

The likelihood of diabetic foot ulcers can be predicted by keeping an eye on a person's feet. A standard method is using a specialized thermometer to check for hotspots on the foot that could be signs of an ulcer. However, there is scant reliable research on the benefits of tracking foot temperature at home[46]. This technique is intended to supplement, not replace, people who regularly check their feet[47].

LIFESTYLE MANAGEMENT

Diet

A healthy diet with some carbohydrates; over time, consuming a consistent quantity of carbohydrates is beneficial to help T1DM patients better control their blood sugar levels. There is insufficient proof that low-carbohydrate diets benefit individuals with T1D[48]. However, it may be possible for some people to follow a low-carbohydrate diet and carefully manage their insulin doses[49].

Exercise

In addition to lowering blood sugar levels, exercise can increase insulin sensitivity and lower the chance of diabetes-related heart disease[50]. Numerous studies have demonstrated that exercise aids glycemic management and reduces HbA1c levels by about 4.2 mmol/mol (0.6%). Studies have shown that individuals with T2D who participate in both physical activity and dietary changes have a lower risk of developing impaired glucose tolerance[51]. Physical activity has an impact on T1D glucose management in that near-exercise energy expenditure rises to account for possible hypoglycemic episodes; this may help to explain why blood glucose levels do not fall during exercise. Exercise increases the translocation and expression of glucose transporter type 4 (GLUT4). This insulin-regulated glucose transporter provides glucose to muscle and adipose cells, making those with T1D more susceptible to nocturnal hypoglycemic episodes[52]. Although exercise may not directly lower blood glucose levels in people with T1D, many benefits remain, such as reduced risk of cardiovascular diseases, improved insulin sensitivity, blood pressure, body composition, lipid profiles, and endothelial function[53].

Medication

The vast majority of drugs for diabetes work by lowering blood sugar levels in various ways. There is widespread consensus that people with diabetes who maintain tight glucose control and keep their blood glucose levels within normal limits have fewer complications, such as kidney or eye problems. Several distinct types of anti-diabetic medications[54]. A "basal-bolus" regimen that most closely mimics natural insulin release is optimal for treating T1D: Long-acting insulin for the basal rate and short-acting insulin with food[55]. Most people with T2D are treated with oral medications, though some ultimately need to be treated intravenously with insulin or GLP-1 agonists. Metformin is usually recommended as the initial therapy for T2D because there is strong evidence that it lowers mortality rates. Furthermore, it decreases the volume of glucose produced in the liver while increasing the quantity of glucose retained by peripheral tissue[56].

THERAPEUTIC PERFORMANCE OF DIABETES MILLETUS EMPLOYING SPECIAL TARGETS

Even though biomedicine has made a lot of progress and we are learning more and more about how to treat different diseases, diabetes is still hard to treat. To solve this problem, researchers from various fields are looking for a way to treat diabetes that is both safe and easy[57]. In addition, rigorous evaluation of the drug action mechanisms of known compounds is beneficial for further validating several novel molecular drug targets.

In contrast to several extant synthetic medicines, natural biomolecules have a wide range of structural variability and have emerged as a valuable source of active agents for developing newer lead compounds in drug discovery[58]. Antidiabetic medications like dipeptidyl peptidase 4, thiazolidinedione, sulfonylurea, or metformin inhibitors are currently used to treat DM. However, these drugs cannot entirely limit diabetes, and future studies are required to find a better cure[59].

In biological systems, receptors are chemical structures composed of proteins that receive and transmit signals. These are some of the receptors and medications that are currently used to treat diabetes, including thiazolidinediones, gliptins, GLP-1, glinides, biguanides, insulin, peroxisome proliferator-activated receptors (PPARs), sulphonylureas, β-glucosidase inhibitors, amylin mimics, SGLT-2, and dopamine D-2 agonists[60]. Pro-hormone convertases (PC I and PC 2) and exoprotease carboxypeptidase make insulin from pro-insulin. These enzymes produce insulin and C-peptide[61]. Insulin faci -litates the translocation of GLUT4 to the cell, causing adipose/skeletal muscle cells to consume additional glucose. Other cutting-edge candidates for managing DM include components of G protein-coupled receptor (GPCR) 119, G protein-coupled estrogen receptor (GPER), vaspin, metrnl, pigment epithelium-derived factor (PEDF), GPCR, GIP, melatonin (MLT), visfatin, ACRP 30 (AdipoQ), and fetuin-A[60,62].

Exenatide and liraglutide are two examples of GLP-1 analogs that replicate the effects of endogenous GLP-1. They contribute to better blood glucose control and weight management by increasing glucose-dependent insulin secretion, decreasing glucagon secretion, delaying stomach emptying, and promoting satiety[63]. Recent randomized controlled trials have shown that T2D patients who consume GLP-1 analogs experience significant weight loss and a significant drop in HbA1c levels. Liraglutide, for instance, was associated with a 13% relative risk decrease in major cardiovascular events, according to the LEADER trial[64]. Frequently observed adverse effects encompass gastrointestinal issues, while ongoing investigations are being conducted to ascertain the long-term safety implications, particularly concerning pancreatitis and thyroid cancer[65].

PPARs, especially PPAR-gamma agonists like pioglitazone, regulate glucose and lipid metabolism and increase insulin sensitivity in peripheral tissues. In diabetic patients, PPAR-gamma agonists have decreased insulin resistance, lowered HbA1c levels, and minimized cardiovascular risk[66]. The administration of pioglitazone has been correlated with weight gain and elevated susceptibility to heart failure, necessitating the meticulous selection of patients[67].

Although vaspin has gained attention recently, its therapeutic applications are not as well established as those of GLP-1 and PPARs. Several investigations have indicated changes in vaspin levels in individuals with diabetes. However, the precise therapeutic consequences of these alterations have not been completely clarified[68]. Further investigation is required to elucidate the mechanisms by which vaspin may be selectively manipulated to provide therapeutic advantages in diabetes. Concurrently, active clinical trials are being conducted to explore this potential. These targets may represent the diabetes treatments of the future (Table 1).

TRADITIONAL PRINCIPLES FOR TREATING DM

Conventional targets are medications used in the market for a while to treat diabetes. Still, their availability is restricted, and they come with several drawbacks, like weight gain, hypoglycemia, and other side effects. They work by keeping the level of glucose in the blood steady. For example, biguanides reduce the amount of glucose produced and increase the amount of glucose used by skeletal muscles and the liver. SGLT-2 antagonists stimulate the kidney's ability to excrete glucose. A-glucosidase inhibitors reduce intestinal uptake of glucose and free fatty acids (FFA). Pancreatic insulin production and sensitivity are both improved by sulphonyl urease. The release of FFA from adipose cells is reduced by 2,4-thiazolidinediones[32,69].

GOALS RECENTLY ACHIEVED IN DM

MLT

The pineal gland secretes the neuroendocrine hormone MLT at night. MLT has been identified as a possible therapeutic target for treating T2D because it also regulates glucose and the pancreatic release of insulin. It exerts its pharmacological effects by interacting with the MT1 and MT2 MLT receptors[70]. Recent research has revealed that mice lacking the MLT MT1 receptor exhibit increased insulin resistance and glucose intolerance. MLT's MT1 receptor is an important therapeutic target for controlling blood sugar levels[71].

PPARs

Transcription factors control gene expression called PPARs, of which there are three types: PPARα, PPARγ, and PPARβ/δ [72]. Thiazolidinediones, PPAR-agonists, turn on the receptor, making the body more sensitive to insulin. After being turned on, they lower the levels of FFA in the blood and change the levels of adipokines. This is possible by increasing

Table 1 Anti-diabetes medications authorized by the Food and Drug Administration

Type of agents	Dosing	Formulation	FDA clearance date	Observations
Euglycemics: Drugs that lower blood sugar levels to typical levels. These drugs shouldn't result in glucose				
Biguanides: Reduces intestinal glucose absorption and hepatic glucose release and enhances insulin sensitivity (increases glucose uptake and utilization)				
Metformin: Glumetza Fortamet®, Glucophage XR®, Glucophage®	500 mg, 1000 mg. 500 mg, 750 mg pills. 500 mg, 750 mg pills. 500 mg, 850 mg, and 1000 mg pills	Initial dose: 500 mg once daily. Dose: 2-3 times a day. Range: 500-2550 mg. Initial: 500 mg 2 times daily or 850 mg once a day	December 1994	SE: Can't use it if you have problems with your liver or kidneys, take medicine for heart failure, or drink too much alcohol. Consume with food (ER with evening meal) 0.03 cases per 1000 individuals are lactic acidosis. Gastrointestinal complaints (3%) such as diarrhea, nausea, and upset stomach
Thiazolidinediones, also known as glitazones or TZDs, are compounds that lower the body's insulin intolerance (muscle and fat tissues)				
Rosiglitazone: Avandia®	Tablets of 2 mg (pink), 4 mg (orange), and 8 mg (red-brown)	Initially: 4 mg per day. Range: 4-8 mg. Take it once or twice every day	May 1999	SE: Bone loss and fractures in women, anemia, edema from fluid retention, weight increase, macular edema (in the eye), and may raise the chance of heart issues, such as angina or heart attacks, which are caused by the heart (myocardial infarction) may lead to or exacerbate cardiac failure. You cannot use this without severe heart failure or liver disease. Liver surveillance is necessary
Pioglitazone (preferred over rosiglitazone): Actos®	Tablets, 15 mg, 30 mg, and 45 mg (white to off-white)	15-30 mg initially; 15-45 mg daily. Dose: One dose per day	July 1999	SE: Bone loss and fractures in women, anemia, edema from fluid retention, weight increase, macular edema (in the eye), and may lead to or exacerbate cardiac failure. You cannot use this without severe heart failure or liver disease. Liver surveillance is necessary
GLP-1 analogs: Make more insulin, stop the liver from releasing glucose after meals, keep the stomach from emptying as quickly, and make people feel full				
Dulaglutide: Trulicity®	1.5 mg or 0.75 mg each time. Under the epidermis (subcutaneous/SQ), injected available in single-dose, dose-specific pen instruments	At first: 0.75 mg once per week. Range: If the reaction is insufficient, it may be increased to 1.5 mg once weekly	September 2014	SE: Sickness, diarrhea, throwing up, stomach pain. It can't be used if you have multiple endocrine neoplasia syndrome type 2 or if you have a family history of medullary thyroid cancer (MEN2). In patients with a history of medullary thyroid cancer, it is contraindicated; there have been a few cases of pancreatitis (inflammation of the pancreas)
Albiglutide: Tanzeum®	30 mg or 50 mg each time under the epidermis (subcutaneous/SQ), injected calls for rebuilding available in single-dose pens with a particular dose	Initial: 30 mg once weekly. Range: Can increase to 50 mg once weekly if inadequate response	September 2014	SE: Upper respiratory infection, nausea, and injection site response. Infrequent cases of pancreatitis (inflammation of the pancreas); contraindicated in patients with a history of medullary thyroid cancer
SGLT2 inhibitors: Make people pee out more glucose				
Dapagliflozin: Farxiga®	5 mg tablets are yellow and round, and 10 mg tablets are yellow and diamond-shaped	5 mg once every day at first. Up to 10 mg per day	January 2014	SE: Increased urination, UT infections, genital yeast infections, dizziness, lower blood pressure, increase in blood potassium; rare severe allergic reactions (severe rash; swelling of the pharynx tongue, body or face) (swelling of the tongue, throat, face or body; severe inflammation). If you have kidney difficulties, you cannot use this product
Empagliflozin: Jardiance®	Tablets 10 mg (pale, beige, round) and 25 mg (pale, beige rectangular)	Initial: 10 mg once daily. Range: Up to 25 mg daily	August 2014	SE: Rare severe allergic responses; side effects including frequent urination, low blood pressure, dizziness, genital yeast infections, and urinary tract infections; and a rise in blood potassium (swelling of tongue, throat, face, or body; severe rash). Do not take it if you have renal disease
Canagliflozin: Invokana®	Tablets come in two different	At first: 100 mg every day.	March 2013	SE: Side effects include frequent or urgent

	dosages and pill colors: 100 mg (colored yellow) and 300 mg (colored white)	Range: 100-300 mg per day. Dose: One dose per day		urination, low blood pressure, dizziness, genital yeast infections, UTIs, a rise in blood potassium, and severe but uncommon allergic reactions (swelling of the tongue, throat, face, or body, severe rash). Do not take it if you have renal disease
DPP-4 inhibitors: Increased insulin production and decreased post-meal liver glucose release are two effects				
Linagliptin: Tradjenta®	5 mg (red-light) tablet	At first, 5 mg every day. Dose: One dose per day	May 2011	SE: No weight gain, nasal congestion, throat pain, rare reports of pancreatitis, extremely rare severe allergic reactions
Saxagliptin: Onglyza®	2.5 mg tablets are pale to light yellow, and 5 mg tablets are pink	Range: 2.5-5 mg daily, starting with 2.5 or 5 mg. Dose: One dose per day	July 2009	SE: Headache, urinary tract illness, and upper respiratory infection. No gaining weight: If kidney issues exist, lower amounts are used
Sitagliptin: Januvia®	Tablets of 25 mg (pink), 50 mg (light brown), and 100 mg (beige)	At first, take 100 mg every day. Daily dose: 25-100 mg. Dosage: Once every day	December 2006	SE: Symptoms include a runny nose, upper respiratory infection, and uncommon severe allergic responses (swelling of the tongue, throat, face, or body, severe rash). There has been no weight increase. If there are kidney issues, lower doses are used
Alogliptin: Nesina®	Tablets of 6.25 mg (light pink), 12.5 mg (yellow), and 25 mg (light red)	Every day, take 25 mg by mouth. Given once a day	January 2013	SE: Upper respiratory infection, headache, sore throat, stuffy or runny nose, uncommon serious allergic responses (swelling of the tongue, throat, face, or body), and severe rash. Accounts of pancreatitis are uncommon. No weight increase
α-glucosidase inhibitors: STARCH blockers are substances that slow down the digestive process and the assimilation of carbohydrates				
Acarbose: Precose® various generics	Tablets of 25 mg, 50 mg, and 100 mg	Initial: Three times per day, 25 mg, 75 to 300 mg. Maximum 150 mg if under 60 kg. Dose: Three times per day	September 1995	SE: Defecation. Take with the first mouthful of your meal. To avoid GI intolerance, begin with a modest dose and gradually increase it
Stimulators of insulin release (insulin secretagogues): Raise the amount of insulin the liver produces				
Glinides				
Nateglinide: Starlix®	Tablets of 60 mg (pink) and 120 mg (yellow)	120 mg three times every day at first (if A1C is close to goal, use 60 mg). Range: 180-360 mg daily dosage is three times	December 2000	SE: Syndrome of uncontrolled hypoglycemia protection for the aged. Only 2 h of actual playtime are involved. Take it within 30 min of dinner
Repaglinide: Prandin®	Tablets of 0.5 mg (white), 1 mg (yellow), and 2 mg (red)	Starting dose: 1-2 mg daily (0.5 mg if A1C 8%). From 0.5 to 16 mg. The maximum dose is 4 mg per dinner. Given twice, three times, or four times per day	December 1997	SE: Hypoglycemic. It is safe for older adults. The activity lasts only 4 h. Take 15-30 min after eating
SFUs				
Glimepiride: Amaryl® various generics	Tablets ranging from 1 mg to 4 mg	To start, try 1-2 mg once a day. Between 1 and 8 mg. One daily dose is recommended	November 1995	SE: Weight increase and hypoglycemia. Only one daily dose is necessary
Glyburide, micronized: Glynase PresTab® various generics	Tablets with dosages of 1.5 mg, 3 mg, 4.5 mg, and 6 mg	Initial: 1.5-3 mg/d; permitted range: 0.75-12 mg. Dosage: One or two daily doses (if > 6 mg)	March 1992	SE: Weight increase and hypoglycemia
Glyburide: Micronase®, DiaBeta® various generics	Tablets of 1.25 mg, 2.5 mg, and 5 mg	Initial: 2.5-5 mg everyday. Range: 1.25-20 mg. To be consumed once or twice every day	May 1984	SE: Hypoglycemia and obesity are possible side effects
Glipizide: Glucotrol®, Glucotrol XL® various generics	Tablets of 5 mg and 10 mg. Tablets of 2.5 mg, 5 mg, and 10 mg ER	At first, 5 mg every day. From 2.5 to 40 mg (20 mg for XL). Dosage: once or twice daily (if more than 15 mg)	May 1984. April 1994	SE: Hypoglycemia and weight increase are symptoms of SE. SFU is preferred by the aged. ER means extended-release/once-daily
Oral pills in combination				

Empagliflozin/metformin, Synjardy®	12.5 mg/500 mg (pale brownish purple), 12.5 mg/1000 mg (dark brownish purple), 5 mg/500 mg (orange-yellow), 5 mg/1000 mg (brownish yellow). Tablet with an oval sheet coating	Starting dose: 5 mg/500 mg or 5 mg/1000 mg. Maximum dose: 25 mg/2000 mg split into two doses	January 2015	SE: It's the same deal with empagliflozin and metformin
Empagliflozin/linagliptin, Glyxambi®	Triangular pills, 10 mg/5 mg (pale yellow), 25 mg/5 mg (pale pink)	At first: 10 mg/5 mg once every day. Range: 5 mg once every day up to 25 mg	February 2015	SE: All the same applies to empagliflozin and linagliptin
Dapagliflozin/metformin XR, Xigduo XR®	10 mg/500 mg (pink), 10 mg/1000 mg (pink to dark pink), and 5 mg/500 mg (orange) (yellow to dark yellow) oval tablets covered in celluloid	Starting dose: The patient's present regimen up to 10 mg/2000 mg per day	October 2014	SE: Dapagliflozin and metformin are the same as previously mentioned
Canagliflozin/metformin, Invokamet®	Film-coated capsule-shaped pills, 50 mg/500 mg (white), 50 mg/1000 mg (beige), 150 mg/500 mg (yellow), and 150 mg/1000 mg (purple)	Beginning: With 50 mg/500 mg or 50 mg/1000 mg. Range: 300 mg to 2000 mg. Taken in 2 divided quantities	August 2014	SE: Identical to the preceding, but with metformin and canagliflozin
Alogliptin/pioglitazone, Oseni®	The next round of pills is available: 25 mg/45 mg (red), 25 mg/30 mg (peach), 25 mg/15 mg (yellow), 12.5 mg/15 mg (pale yellow), 12.5 mg/30 mg (pale peach), 12.5 mg/45 mg (pale red)	Initial dosage: Once daily, 12.5/15 mg. Range: 25/45 mg and higher ingested with or without food once daily	January 2013	SE: The same applies to pioglitazone and alogliptin
Alogliptin/metformin, Kazano®	Oblong pills, 12.5 mg/1000 mg (pale yellow), 12.5 mg/500 mg (pale yellow)	At first: 12.5 mg/500 mg once or twice every day. Maximum range: 25/2000 taken with meals twice a day	January 2013	SE: Alogliptin and metformin in the same way as previously
Linagliptin/metformin, Jentadueto®	Oval pills with dosages of 2.5 mg/1000 mg (light pink), 2.5 mg/850 mg (light orange), and 2.5 mg/500 mg (golden yellow)	Initial dosage: 2 times a day with food, 2.5 mg/500 mg. Range: Twice daily dosages of up to 2.5 mg/1000 mg food	January 2012	SE: With linagliptin and metformin, the same as above
Sitagliptin/metformin, Janumet XR®	Oval pills, 50 mg/500 mg (light blue), 50 mg/1000 mg (light green), and 100 mg/1000 mg (blue)	At first: 100 mg/1000 mg every day. Maximum daily dose: 100 mg/2000 mg. Dosage: Once every day	February 2012	SE: As with sitagliptin and metformin, the same rules apply
Saxagliptin/metformin XR, Kombiglyze XR®	Capsule-shaped pills contain 2.5 mg/1000 mg (pale yellow to light yellow), 5 mg/1000 mg (pink), and 5 mg/500 mg (golden brown to brown)	Starting dose: 5 mg/500 mg or 5 mg/1000 mg once daily. Maximum dose: 5 milligrammes/2000 mg. Dosage: Once every day	November 2010	SE: The same holds for metformin and saxagliptin

SE: Represent potential side effects of the drug. SFUs: Sulfonylureas; TZD: Thiazolidinediones; GLP: Glucagon-like peptide; DDP: Dipeptidyl peptidase; UTI: Urinary tract infection; ER: Extended release; FDA: Food and Drug Administration.

insulin release from the pancreas, improving glucose intake in skeletal muscle and adipose tissues, and lowering glucose synthesis in the liver[73].

G-protein coupled receptor 119

Muscles, liver, and pancreatic beta cells all contain G-protein coupled receptor 119 (GPR119). Like incretin hormones, the activation of GPR119 may increase insulin production and favor insulin secretion when agonists are attached to its binding site[74]. GPR119 improves glucose homeostasis through two distinct mechanisms: The release of GLP-1 and GIP from enteroendocrine cells and the direct impact of the glucose-activated insulin release in β-cells[75].

Glucose-dependent insulinotropic polypeptide

One of the incretin hormones, or GIP, is found in the brain, fatty tissue, and β-cells. It enhances the insulin response prompted by the post-prandial rise in glycemia, where it plays a significant part in T2D and other metabolic disorders [76]. By binding to the GIP receptor, GIP exerts its insulinotropic effects by raising intracellular (cAMP) levels. PKA & exchange protein-activated cAMP2 are activated by elevated cAMP (EPAC2)[77]. The depolarization of the voltage-gated Ca^{2+} channels increase the concentration of Ca^{2+} within the cell, which in turn initiates the release of Ca^{2+} from intracellular stores through PKA and EPAC2. The elevation in Ca^{2+} concentration stimulates the transcription of the pro-insulin gene, which, in turn, contributes to an increase in the amount of insulin secreted by β-cells[78].

Free fatty acid receptor 1

Free fatty acid receptor 1 (FFA1), also known as GPR-40, is a free fatty receptor. FFA1 is primarily present in pancreatic and intestinal cells. Researchers found that the FFA1 receptor affects lipid and glucose metabolism and boosts insulin release from pancreatic β-cells *in vivo* studies using mouse islets' β-cell lines. FFA1 impacts blood glucose levels by indirectly stimulating insulin release from pancreatic cells and increasing incretin hormones directly[79].

FUTURE GOALS FOR DM

Gene therapy

Although very little knowledge exists about these targets' roles in diabetes, they can be critical in managing the disease. A new technique for treating DM is gene therapy, which works by repairing or correcting the defective genes that cause the disease[80]. This approach allows for the replacement of the insulin gene and the transfer of genes *via* viral vectors and non-viral transduction methods to suppress auto-reactive T cells and prevent the destruction of islet cells. According to research, stem cells can be used to treat diabetes because they can readily multiply in culture and act as surrogate β-cells.

Additionally, research has discovered that rodents receiving intrahepatic injections of modified stem cells have low blood glucose levels (Table 2)[81]. Under a fluorescent microscope, the stem cells fluoresce green after the mice have been slaughtered for histopathological examinations. Insulin was found using an anti-human insulin polyclonal antibody to stain the tissue[32]. Mesenchymal stem cells successfully expressed human insulin and maintained blood glucose levels normal, according to a 42-d study. Compared to rodents that were not treated with gene therapy. As a result, as a developing novel technology, genetic treatment has the potential to be used to treat DM[82].

Leukocyte antigen-related tyrosine phosphatase & protein tyrosine phosphatase 1B

Leukocyte antigen-related tyrosine phosphatase and protein tyrosine phosphatase 1B (PTP1B) are critical players in the regulation of insulin signal transductions. An essential stage in the insulin signaling process is tyrosine phosphorylation in the insulin-receptor activation loop[59]. Insulin signaling is negatively regulated by PTP1B, which dephosphorylates phosphor-tyrosine residues in insulin receptor kinase activation regions. More evidence shows that PTP1B, insulin sensitivity, obesity, and T2DM are all linked. PTP1B is also an essential part of the growth of β-cells in the pancreas[83]. For example, Teimouri *et al*[84] reported that PTP1B knockout mice have more cells, and more insulin is released when glucose is present. There is a lot of evidence from these studies that PTP1B plays a role in diabetes. This has sparked much interest in PTP1B inhibitors and the developing and discovery of several PTP1B inhibitors. There are other places where you can find more information about the reported PTP1B inhibitors.

11 beta-hydroxysteroid dehydrogenase

Cortisone, a glucocorticoid, is converted to cortisol, a hormone, by hydroxysteroid dehydrogenase. There are two isoforms of it presently available: 11 beta-hydroxysteroid dehydrogenase 1 (11β-HSD1) and 11β-HSD2. According to research, high blood amounts of glucocorticoids may lead to glucose intolerance, so maintaining 11β-HSD1 levels naturally improves insulin sensitivity[60]. According to one study, inhibiting the 11β-HSD1 may improve insulin sensitivity by reducing insulin resistance and controlling the insulin signaling transduction system. When all the information listed above is considered, 11β-HSD1 emerges as a new molecular target for DM treatment (Table 2)[85].

Fetuin-A

A glycoprotein called fetuin-A is made in the liver and released into the bloodstream. The main protein needed to transport FFA to the bloodstream is fetuin-A. It also plays a role in-cell irritation and degeneration in the pancreas. Tyrosine kinase is a crucial enzyme for insulin signaling that thoroughly opposes insulin activity and is inhibited by fetuin-A (Table 2)[86]. Tyrosine kinase and insulin work together to maintain a healthy blood sugar level. If the blood's fetuin-A content rises, it may lead to insulin resistance and eventually diabetes. Studies have shown that rodents with fetuin-A knockout genes have increased insulin sensitivity, demonstrating the negative relationship between fetuin-A and insulin sensitivity in diabetes[87].

Serpin A12 or vaspin

Serpin A12, also known as vaspin, is a glycoprotein in serum that belongs to the superfamily "serpin". It is derived from fatty cells and significantly impacts insulin activity. It has been discovered that as the severity of diabetes rises, the serum levels of vaspin begin to fall. This raises the possibility that increasing the vaspin levels in circulation could aid in managing DM[88]. Vaspin administration in rodents has been linked in studies on rodents to enhanced glucose tolerance and insulin sensitivity. This implies that it might be an option for therapy for managing metabolic disorders such as T2D and obesity. Vaspin can exert its effect by suppressing the insulin-degrading enzyme known as kallikrein 7 (KLK7), which in turn reduces the insulin's half-life and causes the insulin to be degraded more quickly (Table 2)[89]. Because KLK7 is blocked, insulin signals work better, and insulin's half-life is lengthened, which helps lower blood glucose levels. It also does a few other things that indirectly reduce blood sugar. For example, it makes you eat less, which lowers your hepatic glucose production *via* increasing insulin signaling in the liver and reducing hepatic lipid accumulation[90]. It decreases inflammation and boosts insulin signaling in brown adipose tissue and white adipose tissue. It activates the vagus nerve in the central nervous system to reduce appetite[91].

Table 2 A list of recently developed novel anti-diabetic targets and their method of activities

Nature	Special targets	Diabetics	Method of activity	Ref.
Gene	Gene therapy	Auto-reactive T cells need to be stopped from killing islet cells	Act by fixing or modifying the problematic genes	[81]
Glycoprotein in serum	SERPIN A12 or vaspin	KLK7 reduction enhances insulin signaling and lengthens the half-life of insulin, contributing to lower blood sugar levels	Vaspin blocks KLK7	[89]
Adipokine	Metrnl	Enhanced insulin responsiveness	Cause of PPAR pathway upregulation	[92]
Hormone	ACRP-30	Acrp30 increases insulin sensitivity and lowers blood sugar	Low amounts bring on insulin sensitivity	[106]
Glucocorticoids	11β-HSD1	11β-HSD inhibition glucose reduction, insulin sensitivity improvement	Increasing amounts lead to glucose sensitivity	[85]
Glycoprotein	Fetuin-A	When fetuin-A levels are low, insulin sensitivity will go up	Associated with beta-cell inflammation	[86]
Glycoprotein	GPER	Boost insulin production	Through binding with Gi/o and Gs proteins, glucose homeostasis is regulated	[94]
Glycoprotein	PEDF	Insulin sensitivity is improved by reducing PEDF levels	Insulin resistance is caused by an upregulated chain of kinase-mediated Serine/threonine phosphorylation of IRS	[99]
Protein	Visfatin	Activity that mimics insulin	Receptor for insulin that it binds to	[103]
Protein	CCN3/NOV	Improved glucose tolerance and insulin sensitivity	Strong correlation with hs-CRP	[97]
Glycoprotein	PTP1B		Inhibits insulin signaling by dephosphorylating insulin receptor kinase	[83]

KLK7: Kallikrein 7; PPAR: Peroxisome proliferator-activated receptor; 11β-HSD1: 11 beta-hydroxysteroid dehydrogenase 1; GPER: G protein-coupled estrogen receptor; PEDF: Pigment epithelium-derived factor; CCN3/NOV: Cellular communication network 3/nephroblastoma overexpressed; PTP1B: Protein tyrosine phosphatase 1B; hs-CRP: High-sensitivity C-reactive protein.

Metrnl

Metrnl is an adipokine derived from the fatty tissues prevalent in the body's subcutaneous white fat. Metrnl is crucial for sustaining immunological inflammation, cardiovascular function, lipid metabolism, energy metabolism, insulin sensitivity, and its essential role in maintaining glucose homeostasis (Table 2)[92]. According to a report, researchers discovered that it functions by upregulating the PPAR pathway, which increases insulin sensitivity in mice models. Additionally, it has been found to encourage the browning of adipose tissue, increasing energy expenditure and better glucose tolerance[93].

GPER

GPER is an orphan 7-transmembrane G-protein-coupled estrogen receptor that helps send signals about estrogen. They are found in the intracellular membranes of cells. Gi/o and Gs protein binding in organisms are crucial for controlling glucose homeostasis[94]. It was discovered that a GPER-deficient female mouse model produces insufficient insulin, leading to DM. A study also found that estrogen levels are high in premenopausal women, which benefits glucose homeostasis, lipid metabolism, and blood pressure[95]. In addition to decreasing inflammation, estrogen levels decline after menopause, making the female population more susceptible to metabolic disorders and insulin resistance, contributing to DM[96]. This data suggests that GPER may be essential for managing diabetes and a valuable drug target for treating diabetes and associated disorders (Table 2).

Cellular communication network 3/nephroblastoma overexpressed

Cellular communication network 3 (CCN3), known as nephroblastoma overexpressed, is a protein high in cysteine with growth-regulating properties. Numerous human organs and bodily fluids, including the musculoskeletal system, kidneys, and cerebrospinal fluid, have been found to contain them[59]. Hyperlipidemic obese patients have substantially higher than expected plasma levels of CCN3, correlated with high-sensitivity C-reactive protein, body mass index, and fat mass. Dalle *et al*[97] showed that mice who didn't have enough CCN3 and ate standard high-fat diets lost much weight and had better glucose tolerance and insulin sensitivity (Table 2). Furthermore, Li *et al*[98] compared serum CCN3 levels in recently diagnosed T2DM (nT2DM) patients to healthy control subjects. CCN3 levels were significantly higher in T2DM individuals.

PEDF

The serine protease inhibitor family includes the 50 kDa PEDF, secreted from adipose tissue and the pigment cells of the

human eye. It induces the insulin receptor substrate to undergo kinase-mediated serine/threonine phosphorylation, which results in decreased insulin signaling and insulin resistance in body cells (Table 2)[99]. Additionally, the body's insulin sensitivity causes the production of interleukin-1 and tumor necrosis factor-alpha (TNF-α) in the system. The research discovered that animals' insulin sensitivity decreased after receiving PEDF but returned to normal after receiving anti-PEDF. PEDF correlates well with insulin resistance in infants and adults[100]. Therefore, if we can lower the amount of PEDF in the blood, it might help the body respond better to insulin. This makes PEDF a possible new way to treat DM and other metabolic syndromes[81].

Visfatin

Visfatin is a protein with many different functions. It is also called nicotinamide phosphoribosyl-transferase. It was founded in 2005. It can be found in several organs and tissues, but most comprise visceral adipose tissue. It has insulin-like properties, which means it helps to restore insulin sensitivity. This suggests that it may also play a role in diabetes, making it a new way to treat DM[101]. It has been demonstrated that serum visfatin concentrations rise alongside the progression of T2DM, establishing a relationship between visfatin and T2DM. Current research has shown that visfatin binds to the insulin receptor at a location different from that of insulin, suggesting that it has properties similar to insulin and stimulates cell growth[102]. Though scientists are investigating the underlying mechanisms of visfatin in DM, it is unclear how visfatin is fully linked to the disease. Nevertheless, there are some visfatin stimulators and inhibitors. With this knowledge, it is possible to conclude that visfatin and diabetes are related in the body, making it an appropriate focus for DM treatment (Table 2)[103].

ACRP 30

ACRP 30 or Adipocyte complement protein of 30 kDa, the capacity of adipose tissue to store fat has long been known. However, current studies have demonstrated that it may serve as a reservoir of hormones such as Acrp30, adiponectin, resistin, TNF-α, leptin, or adipsin[104]. TNF-α is a crucial pro-inflammatory mediator responsible for insulin resistance, and serum protein Acrp30 is found to serve a primary part in managing DM. In addition, another report reveals that Acrp30 levels are reduced in numerous obesity and diabetes models[105]. Since mice missing Acrp30 exhibit insulin resistance, which results in the development of DM, high TNF-α levels also demonstrate a negative correlation of this protein with DM (Table 2)[106]. When the concentration of Acrp30 in the blood is raised, insulin sensitivity can also be elevated, making it easier to control blood glucose levels. As a result, Acrp30 will potentially become an additional avenue for the therapy of DM[107].

IMPACT OF SOCIAL DETERMINANTS ON DM

Current literature increasingly underscores the substantial influence of social determinants on the development, management, and outcomes of DM. This section aims to provide an updated perspective on this critical aspect, incorporating recent research findings and insights. Recently, studies have reaffirmed the strong association between socioeconomic status (SES) and the prevalence of diabetes. A study by Liu *et al*[108] highlighted a significant correlation between lower SES and a higher risk of developing diabetes. This socioeconomic gradient in diabetes incidence has been consistently observed in diverse populations.

Data from Tapager *et al*[109] emphasize the role of healthcare access in diabetes management. Their findings indicate that individuals with limited access to healthcare services face more significant challenges in managing their diabetes, resulting in health disparities. This observation aligns with the growing awareness of differences in diabetes outcomes based on factors such as race and geography. Moreover, research published by Kanchi *et al*[110] has shed light on the significance of the food environment in diabetes risk. Their study demonstrated that neighborhoods with limited access to fresh and healthy food options were associated with higher rates of diabetes incidence. This highlights the importance of addressing the food environment as a key social determinant in diabetes prevention and management.

The latest research on psychosocial factors and how they affect diabetes control has revealed significant new information. According to a study by Abate and Gedamu[111], stress and social support networks significantly impact how well people with diabetes manage their blood sugar levels. These results highlight the importance of comprehensive psychosocial support in treating diabetes. Based on these recent findings, our discussion aims to underscore the evolving understanding of how social determinants intricately shape the landscape of DM. These insights emphasize the need for a multifaceted approach that considers clinical factors and the social, economic, and environmental contexts in which diabetes occurs.

CONCLUSION

DM is a pervasive and challenging health condition affecting a substantial population worldwide. The primary goal of DM therapies is to achieve near-normal blood glucose levels. However, it is essential to acknowledge that current treatments cannot offer a complete cure; they can only manage symptoms and slow the progression of the disease, often accompanied by a range of adverse effects. The quest for innovative solutions to address DM and its consequences is an ongoing endeavor within the scientific community. Researchers are steadfast in their pursuit of compounds that could potentially offer a lasting remedy for DM with minimal side effects. While traditional methods, such as insulin therapy

and biguanides, have been relied upon for an extended period, other classes of medications, including sulphonylureas, glinides, thiazolidinediones, gliptins, inhibitors of α-glucosidase, analogs of amylin, SGLT-2 inhibitors, and dopamine D-2 agonists, have also been explored. Unfortunately, these treatments are not without limitations, often presenting adverse effects ranging from bladder cancer to hypoglycemia and weight gain.

In response to these challenges, researchers have been actively investigating alternative targets for diabetic therapy. While targets like PPARs have garnered significant attention over the past decade, the translation from pre-clinical research to clinical studies and commercialization has been limited. This underscores the pressing need for novel, creative pharmacological targets in diabetes management. In light of recent advancements, several receptors, including GPCR 119, GPER, GPCR, GIP, MLT, visfatin, ACRP 30, fetuin-A, PEDF, metrnl, vaspin, and 11-hydroxysteroid dehydrogenase-1, have emerged as promising candidates that play a direct or indirect role in insulin regulation. These receptors hold the potential to be leveraged as therapeutic targets for diabetes management, paving the way for the development of long-term remedies and the mitigation of its complications. Furthermore, it is worth noting that cutting-edge approaches, such as gene therapy and stem cell-based interventions, hold the promise of delivering treatments with increased efficacy and fewer adverse effects. These innovative strategies represent exciting avenues for exploration in the pursuit of more effective and patient-friendly interventions for DM.

In conclusion, while we acknowledge the challenges associated with the existing approaches to diabetes management, we remain optimistic about the future of diabetes research and therapy. Our understanding of the intricacies of this condition continues to evolve, offering fresh perspectives and novel opportunities. We encourage continued exploration into the receptors and innovative therapies discussed here, anticipating that they will contribute significantly to developing effective, enduring solutions for DM and its associated complications.

FOOTNOTES

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REVIEW

Genetic perspectives on childhood monogenic diabetes: Diagnosis, management, and future directions

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Abstract

Monogenic diabetes is caused by one or even more genetic variations, which may be uncommon yet have a significant influence and cause diabetes at an early age. Monogenic diabetes affects 1 to 5% of children, and early detection and genetically focused treatment of neonatal diabetes and maturity-onset diabetes of the young can significantly improve long-term health and well-being. The etiology of monogenic diabetes in childhood is primarily attributed to genetic variations affecting the regulatory genes responsible for beta-cell activity. In rare instances, mutations leading to severe insulin resistance can also result in the development of diabetes. Individuals diagnosed with specific types of monogenic diabetes, which are commonly found, can transition from insulin therapy to sulfonylureas, provided they maintain consistent regulation of their blood glucose levels. Scientists have successfully devised materials and methodologies to distinguish individuals with type 1 or 2 diabetes from those more prone to monogenic diabetes. Genetic screening with appropriate findings and interpretations is essential to establish a prognosis and to guide the choice of therapies and management of these interrelated ailments. This review aims to design a comprehensive literature summarizing genetic insights into monogenetic diabetes in children and adolescents as well as summarizing their diagnosis and management.

Key Words: Monogenic diabetes; Maturity-onset diabetes of the young; Insulin resistance; Genetic mutation; Beta-cell function

Core Tip: Monogenic diabetes, a rare yet impactful condition in childhood, results from genetic variations, causing early-onset diabetes. Affecting 1%-5% of children, early detection and tailored genetic treatments can enhance long-term health. Culprits include genetic variations in beta-cell regulatory genes and severe insulin resistance. Identifying specific types allows transitioning to sulfonylureas while maintaining glucose control. Tools to differentiate diabetes types underscore genetic screening's importance for prognosis and treatment guidance. This review delves into genetic insights into childhood monogenic diabetes, offering diagnosis and management guidance for affected youth's better health.

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INTRODUCTION

Diabetes mellitus (DM) is a well-known metabolic syndrome characterized by elevated blood glucose levels and its frequently related symptoms, including polyuria and polyphagia. It has the potential to produce substantial medical issues, reducing life longevity and performance of life, and stands as a significant public health concern. For persons born in the United States, the lifetime chance of acquiring diabetes is predicted to be one in three[1]. Types of diabetes are commonly classified as autoimmune-mediated type 1 diabetes, which causes insulin insufficiency; diabetes caused by pancreatic injuries; diabetes caused by particular genetic abnormalities; and type 2 diabetes, characterized by decreased insulin production and resistance to insulin's activities[2,3]. Table 1 below shows the general classification of diabetes.

The types of diabetes that are caused by monogenic alterations are the ones that are better suited to much more specific therapies. There are more than 50 genetic subgroups wherein the transmutation seems unaffected by behavioral or environmental variables. Since monogenic types of diabetes have a recognized origin, their pathophysiological mechanisms are more appreciated adequately than those of other diabetes types. Although these abnormalities constitute a minute percentage of overall diabetes cases (about 1 to 5% of findings in pediatric and young people), they provide a chance to display the practicality of accurate prognostic and treatment procedures[4-6]. Despite the necessity of a precise diagnosis, it is believed that about 80% of overall monogenic diabetes patients stay undiagnosed[7].

A single genetic mutation induces an uncommon kind of diabetes called monogenic diabetes. Gestational diabetes due to a mutation in the glucokinase (GCK) gene, maternally inherited diabetes and deafness (MIDD), mature-onset diabetes of the young (MODY), and other conditions are examples of such mutations. Early-onset diabetes and familial background of diabetes in several first-degree cousins are two characteristics of individuals with monogenic diabetes. Type 1 DM and type 2 DM are common misdiagnoses for monogenic diabetes. In some circumstances, the causal gene can guide the therapeutic strategy, and precise molecular and genetic identification of monogenic diabetes assists in the identification of affected members of the family. MODY is the least frequent subtype of monogenic diabetes among several forms. This is a medically diverse collection of illnesses characterized by cell malfunction, which results in early-onset diabetes and is inherited autosomally[8-10].

One of the primary challenges in diagnosing monogenic diabetes in pediatric patients lies in its clinical and genetic heterogeneity. Currently, the diagnosis often involves a combination of clinical presentation, family history, and genetic testing[11]. However, recent advancements in genetic testing methodologies have significantly improved our ability to identify specific genetic mutations associated with monogenic diabetes. Recent studies have shown promising results using next-generation sequencing (NGS) technologies in identifying monogenic forms of diabetes. These techniques allow for a more comprehensive analysis of the patient's genetic profile, enabling the detection of rare mutations that traditional methods might have missed. In addition to NGS, there is ongoing research into using machine learning algorithms to assist in interpreting genetic data. These algorithms can help clinicians pinpoint potential genetic mutations and streamline the diagnostic process[12-14].

The treatment of monogenic diabetes in pediatric patients is evolving to become more tailored and disease-specific. Understanding the genetic basis of the condition allows for targeted therapies that can address the root cause of the disease[15]. Recent therapeutic advancements include the development of gene-based therapies, such as gene editing techniques like CRISPR-Cas9, which hold promise in correcting genetic mutations responsible for monogenic diabetes. These therapies have succeeded in preclinical studies and may offer a potential cure for certain subtypes of monogenic diabetes[16]. International collaborations and data-sharing initiatives have also enabled researchers to collect valuable information on the global challenges of treating monogenic diabetes in pediatric populations. This collaborative approach fosters the sharing of best practices and the development of innovative treatment strategies[17].

This article aims to provide a comprehensive overview of the complex connections between genetic mutations, clinical symptoms, and treatment approaches in children and teenagers with monogenic diabetes. The article aims to improve the understanding of clinicians, researchers, and healthcare providers by exploring the genetic aspects of this condition. This will help them make informed diagnosis, treatment, and long-term care decisions. Moreover, with the ongoing progress in genetic research, this review article becomes crucial in laying the foundation for enhancing patient outcomes, developing personalized therapeutic strategies, and identifying potential areas for future research and intervention.

Table 1 General classification of diabetes

Type of diabetes	Causes and characteristics
Type 1	Insulin insufficiency is caused by the autoimmune destruction of β-cells
Type 2	Insulin resistance and inadequate secretion of insulin
Gestational	Diagnosed in 2nd or 3rd trimester of pregnancy
Monogenic	Due to genetic variation in one or multiple genes leading to maternally-inherited diabetes and deafness, mature-onset diabetes of the young, and Neonatal diabetes

OVERVIEW OF MONOGENIC DIABETES

Monogenic diabetes encompasses a collection of infrequent hereditary variants of diabetes that arise from mutations occurring in a solitary gene. In pediatrics, monogenic diabetes, or MODY, has been comprehensively screened in many investigations with an estimated frequency of 1.1-4.2 percent[9]. A baseline MODY occurrence of 1.2 percent was found in the United States multicenter population-based study "SEARCH for Diabetes in Youth", and a further 0.2 percent had neonatal diabetes. Monogenic diabetes is 2.5 percent more common in individuals diagnosed in pediatric clinics in the United Kingdom than in patients diagnosed in general demographics over the age of 20 years[10,18]. The mutations could have occurred spontaneously, or they could have been transmitted predominately or recessively. Mutations in only one gene cause monogenic diabetes inherited either dominantly or recessively, or it could be a spontaneous case due to a de novo mutation. Most childhood cases of monogenic diabetes are caused by mutations in the genes that control beta-cell function.

In contrast, mutations causing severe insulin resistance can occasionally cause diabetes. Molecular genetic testing yields a diagnosis in about 1 or 2 percent of cases. Clinicians believed long ago that an abnormally significant heritable mutation could produce diabetes in some people. The observation was made on two primary clinical characteristics indicative of a putative monogenic origin, including diabetes in newborns or neonatal DM (NDM) and family having diabetes in teenagers or early adulthood from many generations, indicating an autosomal dominant inheritance pattern [18].

Maturity-onset diabetes of the young

Monogenic β-cell malfunction is known as MODY and was first clinically diagnosed in the 1970s by examining numerous multigenerational families[19]. MODY is identified by:

Onset at an early age, dominant, autosomal inheritance, No signs of metabolic syndrome, Persistent synthesis of endogenous insulin, Not having β-cell autoimmunity.

MODY accounts for 1%-6% of all diabetes cases, and its prevalence is increasing among children and young individuals. However, it is believed that a significant portion, around 80%, of MODY cases are misdiagnosed as either type 1 or type 2 diabetes[7,20]. Some advanced nations offer molecular genetic diagnostics, utilizing mainly Sanger sequencing, which costs £350 per gene in the United Kingdom as of this writing. The ramifications of molecular diagnosis are significant for both the probands and their families, which will benefit from cascade monitoring and definitive diagnosis as well as from individualized care made possible by molecular diagnosis. GCK, hepatic nuclear factor 4α (HNF4A), hepatic nuclear factor 1b (HNF1B), and hepatic nuclear factor 1α (HNF1A) gene mutations are the leading causes of the most prevalent kinds of MODY (in order of frequency in the United Kingdom)[21,22].

HNF1A-MODY (MODY3)

In the United Kingdom, 52% of all incidences of MODY fall into this category, making it the most prevalent type of monogenic diabetes. Hyperglycemia occurs in the 2nd and 4th decades of life due to genetic abnormalities in the transcription factor HNF1A, which promote increasing β-cell malfunction (Figure 1)[23]. Similar to type 1 and 2 diabetes, expert follow-up is advised since microvascular and macrovascular complications are frequent if glycemic objectives are not met. Sensitivity to sulfonylurea medications is among HNF1A-most MODY's significant characteristics[24]. The very first therapy is the administration of a small number of oral hypoglycemics, such as gliclazide (20-40 mg daily), which may typically be effectively replaced in patients who were previously treated with other medications, such as insulin, without a decline in glycemic control. However, it is important to note that therapy escalation is a common occurrence as individuals undergo treatment, particularly when it comes to insulin usage as β-cell dysfunction continues to deteriorate over time. Extra-pancreatic characteristics of HNF1A-MODY include reduced blood C-reactive protein concentration and a low renal glucose threshold. The latter characteristic, in particular, may serve as a useful diagnostic marker for identifying this condition[21,25].

PDX1 (MODY 4) and NEUROD1 (MODY 6)

Another form of MODY is caused by a defect in insulin gene (INS) promoter factor 1 (IPF1). PDX1 is a transcription factor that contains a homeobox and plays a role in pancreatic development and the expression of INSs. The NEUROD1 mutation is found in a basic-loop-helix transcription factor, which impacts the development of both the pancreas and neurons. Most patients must undergo insulin treatment[2].

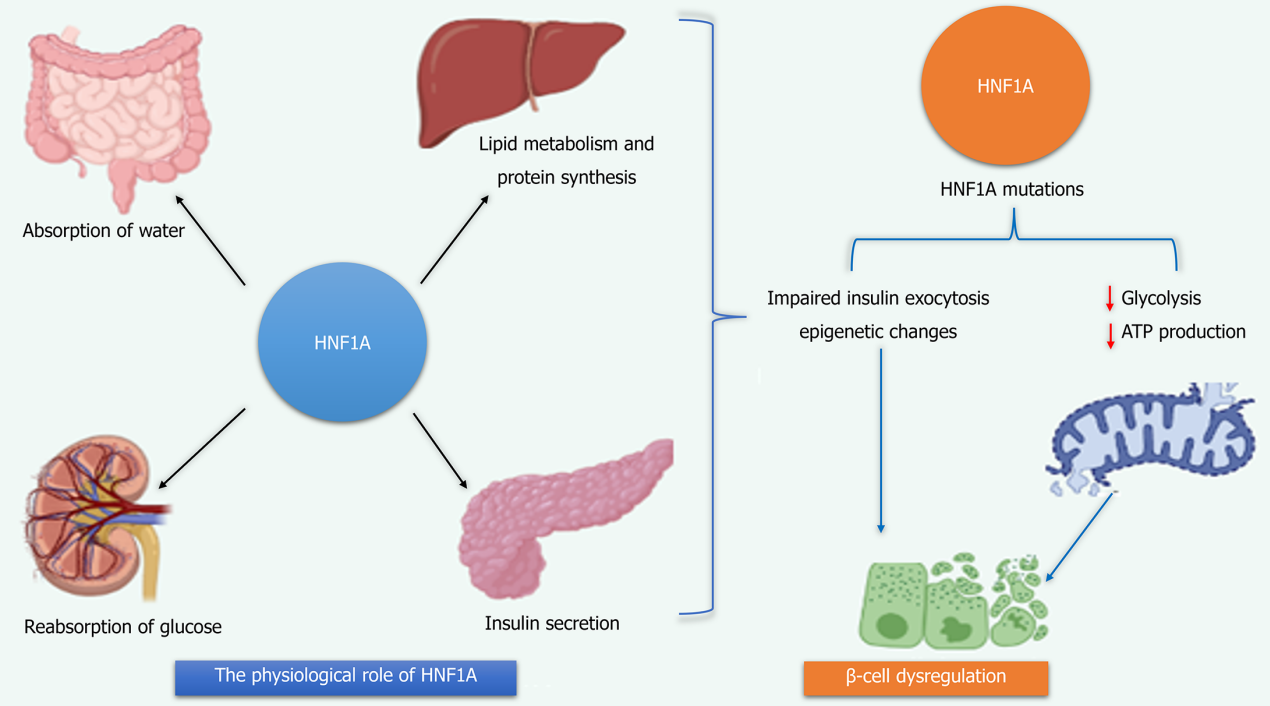

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Figure 1 Hepatic nuclear factor 1α mutations cause beta cell dysfunction. hepatic nuclear factor 1α (HNF1A) controls the expression of genes essential in carbohydrates and protein metabolism, such as glycolysis, the Calvin cycle, and mitochondrial oxidation. HNF1A deficiency inhibits mitochondrial respiration in human embryonic stem cell-derived beta cells. HNF1A: Hepatic nuclear factor 1α.

GCK-MODY

The significant and frequent reason for monogenic diabetes, known as GCK-MODY or non-progressive hyperglycemia associated with GCK, is thought to affect as many as 1 in 1000 people[26]. GCK (β-cell glucose sensor) carries heterozygous inactivating mutations causing GCK-MODY[27,28]. The chain of processes leading to insulin production is initiated by glucose metabolism, triggered by GCK activity. However, when GCK activity is impaired, the threshold glucose level needed to start insulin secretion is raised, even though the β-cell function is relatively unaffected[29,30]. Disorders in such pathways are also brought on by GCK's crucial process in the storage and release of liver glucose. The end outcome is mild fasting hyperglycemia with an A1C of 5.8 to 7.6% (40 to 60 mmol/mol) and a range of 97 to 150 mg/dL (5.4 to 8.3 mmol/L) in most cases[31]. Even though there may occasionally be an age-associated elevation in A1C similar to that reported in elderly populations, this trend is present at birth (congenital). It has remained remarkably steady through time[32]. Patients are asymptomatic and are not detected with hyperglycemia until accidental lab testing or regular monitoring, frequently as pediatric accidental hyperglycemia, throughout pregnancy, or after an incidental illness results in the condition[33-36].

HNF1B-MODY

Diabetes and renal cysts are the two most common features of HNF1B-MODY. However, other developing abnormalities in many systems can also occur[37]. Since the etiology is a deficiency in β-cell growth, this type of diabetes commonly manifests in adolescence or early adulthood, is frequently insulin-dependent, and often requires insulin. There is a decreased pancreatic exocrine function, which may need to be treated. Exocrine pancreatic insufficiency can be diagnosed with the help of a smaller pancreatic tail or low fecal elastase levels. Several developmental kidney diseases have been reported, albeit renal cysts are typically present. The most frequent genetic cause of pediatric kidney disease, which accounts for 20%-30% of cases, is HNF1B-MODY[38].

KCNJ11-NDM and ABCC8-NDM

The most frequent reason for permanent neonatal DM (PNDM) and a significant source of transient newborn (TNDM) is activated heterozygous abnormalities in either gene encoding the subunits of the β-cell ATP-sensitive potassium (KATP) channel (KCNJ11 or ABCC8)[39,40]. However, in the presence of acute hyperglycemia, mutant channels maintain membrane hyperpolarization. However, these deficiencies can be treated with large doses of sulfonylurea, allowing patients to transition from insulin and resume meal-stimulated insulin release with little to no hypoglycemia. However, after more than 10 years of therapy, effective glycemic control frequently lasts[41-43]. When a genetic diagnosis is made, initial sulfonylurea administration could, at least to some extent, alleviate a range of neurological impairments caused by more harmful variations. The medical phenotype is connected with the intensity of the mutation[44,45]. TNDM is commonly caused by gentle stimulatory mutations (ABCC8 more frequently than KCNJ11), or they may manifest as a specific type of MODY in people or families who develop later MODY-like diabetes, which is typically able to respond to

Table 2 Different types of the most common monogenic diabetes and their characteristics

Phenotypes	Responsible gene	Characteristics
HNF1A-MODY	HNF1A	Loss of function of the β-cell transcription factor, glucosuria,
GCK-MODY	GCK	Reduced glucokinase enzyme function, raising insulin secretion setpoint
HNF1B-MODY	HNF1B	Pancreatic/renal transcription factors' loss of functioning, genitourinary/renal malformations, exocrine pancreatic insufficiency, hypomagnesemia, variations in liver function tests, developmental delay, hyperuricemia
KCNJ11-NDM	KCNJ11	Mutation in the β-cell KATP channels' Kir6.2 subunit leads to impaired neuro-developmental dysfunction of insulin secretion
ABCC8-MODY	ABCC8	Mutation in the β-cell KATP channels' SUR1 subunit, leading to impaired insulin secretion and neurodevelopmental dysfunctions
INS-NDM and-MODY	INS	abnormalities in the proinsulin gene leading to a gradual deterioration of β-cell functioning capability due to the accumulation of improperly coiled proinsulin proteins

KATP: ATP-sensitive potassium; NDM: Neonatal diabetes mellitus; MODY: Mature-onset diabetes of the young; INS: Insulin gene; HNF1A: Hepatic nuclear factor 1α; GCK: Glucokinase; HNF1B: Hepatic nuclear factor 1b.

a sulfonylurea and are not identified to have had neonatal hyperglycemia[46,47]. Bi-allelic moderately activated mutations (often homozygous) and compound heterozygous abnormalities, wherein one mutant is stimulating while the other is an impairment form, are two additional uncommon causes of neonatal diabetes from KATP mutations. Nevertheless, neonatal hyperinsulinism is caused by homozygous loss of function mutations in either gene[48,49].

INS-NDM and INS-MODY

A hereditary assessment may not alter the course of therapy for some varieties of monogenic diabetes, and that may nonetheless open the door to a precision-based strategy. For instance, the second most frequent etiology of PNDM is heterozygous abnormalities in the pro INS, which gradually deteriorate β-cell functioning capability due to the accumulation of improperly coiled proinsulin proteins[50]. Even though the only existing therapy is insulin, delaying the gradual decline of β-cell activity and improving long-term consequences may be possible by reducing the stimulation for increased synthesis of the genetically variant nutrients *via* minimizing blood glucose levels by initial intensive insulin administration[51]. Both permanent and transient neonatal diabetes are also caused by nonsense or promoter variations of the INS that inhibit or significantly reduce insulin production. The most effective treatment choices for these uncommon patients have not yet been determined[52-54]. Different types of monogenic diabetes in young are given in Table 2 below.

Monogenetic diabetes in neonates and children

Recent advancements in molecular genetics have provided us with a better understanding of the causes of diabetes at a young age. It has been discovered that these cases are often the result of monogenic abnormalities and mutations in a single gene. NDM is a condition that impacts approximately 1 in every 90000 to 160000 live births[55]. There are more than 20 genetic factors that might develop NDM. One of the most probable etiologies of diabetes is identified before the age of six months; hence, additional clinical factors should be investigated to help direct genetic testing. Medically, two categories could be distinguished: (1) TNDM, which is reversible after a median of 12 wk without the need for further treatment, although up to 50% of patients could recur over the pediatric age range[56,57]; and (2) in addition, lifetime insulin therapies are necessary for people with PNDM after their diagnosis[58]. Mutations in the KCNJ11 gene, which codes for the Kir6.2 subunit of the KATP channel, are the second most prevalent sources of mutations in people with diabetes who are reported well before the age of six months of childhood. These abnormalities might cause either TNDM (10%) or PNDM (5% of cases)[59,60].

GENETIC VARIATIONS ASSOCIATED WITH MONOGENIC DIABETES IN CHILDREN

Monogenetic diabetes due to variations in the ATP-sensitive potassium channels

The KCNJ11 and ABCC8 genes, which code for four pore-forming Kir6.2 subunits and four SUR1 regulatory subunits, create the hetero-octameric complexes that comprise the K_{ATP} channels[61,62]. Every rise in intrinsic metabolic activities causes the cell's ATP /adenosine diphosphate ratio to rise and causes the K_{ATP} channels to shut. Depolarization of the cell membrane, as a result, eventually causes the release of insulin[63]. KCNJ11 or ABCC8 variants are discovered in about 50% of patients having permanent newborn diabetes. Such variations primarily weaken the channel's responsiveness to ATP, thus preventing channel closure and subsequent release of insulin[64]. The particular mutation determines the phenotype, and there is a strong association between the functional intensity of the mutation and the phenotype, with a few notable omissions[65]. Permanent diabetes, instead of transient, is more common in neonates with changes in amino acids of Kir6.2, which is neonatal diabetes induced by KCNJ11 mutations (10%). Diabetes commonly manifests between

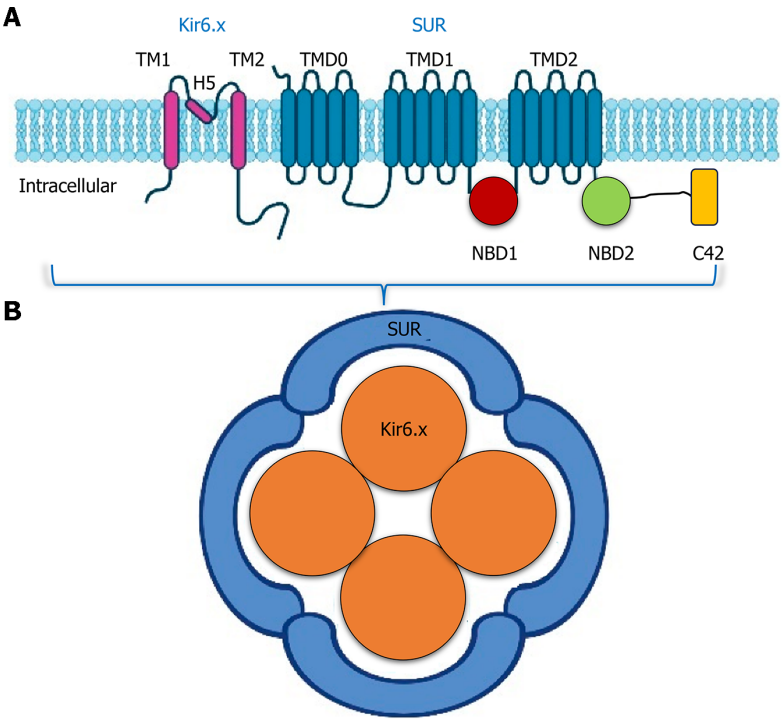

Figure 2 The ATP-sensitive potassium channel exhibits a simple structural composition. A: The Kir6.x subunit consists of two transmembrane regions (TM1 and TM2) linked by a pore-forming area known as H5. The SURx subunit consists of three domains, including TMD0 with five transmembrane sections and TMD1 and TMD2 with six transmembrane regions each. The intracellular localization of the nucleotide-binding domains (NBD1 and NBD2) has been observed. The only distinction between SUR2A and SUR2B resides in their C-terminal end (C42); B: The functioning ATP-sensitive potassium channel comprises four Kir6.x subunits and four SURx subunits. Citation: Clement A, Guo S, Jansen-Olesen I, Christensen SL. ATP-Sensitive Potassium Channels in Migraine: Translational Findings and Therapeutic Potential. Cells 2022; 11. Copyright ©The MDPI. Published by MDPI.

infancy and 26 wk of age but is usually accompanied by severe ketoacidosis (30%) and hyperglycemia[66].

Reduced newborn weight is widespread but lesser in individuals with 6q24 imprinting anomalies. Approximately 20% of probands with permanent neonatal diabetes have related neurological symptoms because the Kat p channel is expressed in nerves and musculature. Individuals sometimes develop a severe syndrome of epilepsy, neonatal diabetes, and developmental disorders [collectively known as or developmental delay, epilepsy and neonatal diabetes (ENDD)][67, 68]. Nevertheless, an intermediary ENDD syndrome is more prevalent and is distinguished by DM and relatively developmental disorders without seizures. Like SUR1 neonatal diabetes, transient neonatal diabetes is more prevalent than permanent, and neurological symptoms are less frequent and typically include speech problems and aberrant breast-feeding behavior[48,69]. K_{ATP}-linked TNDM might reoccur early in adulthood, like in individuals with 6q24 imprinting anomalies. Since oral sulfonylureas (SU) are the most successful treatment for people with activating K_{ATP} channel mutations while being insulin dependent, it is critical to detect these patients. In an ATP-independent way, these attach with the SUR subunit and block the channels[42,70].

More than 90% of people with Kir6.2 diabetes and 85% with SUR1 diabetes can switch from insulin to oral hypoglycemic pills and improve their blood sugar management without an increased glucose level. Furthermore, the quantity required is significantly greater than that used in type 2 diabetes (and slightly lesser in individuals with ABCC8 initiating genetic variation than in those with KCNJ11 mutations) or might result in temporary diarrhea[42]. KCNJ11-activated heterozygous mutations are linked to Kir6.2 DM. Since over 90% of alterations occur "de novo," individuals are typically born to parents without diabetes. Autosomal dominant transmission is evident in familial instances. This means there is a 50% chance of NDM for each subsequent child of an afflicted person. Similar to how few SUR1 DM patients have DM in their families. Most outbreaks also come from de novo heterozygous mutations, and those with mutations have a 50% probability of passing it on to their offspring.

Moreover, recessive inheritance is present in about 40% of PNDM individuals with ABCC8 mutations[71]. The probability of newborns' diabetes in these situations is 25% for every sibling of the children, but the affected child has a very minimal possibility of shifting the condition onto offspring. Nevertheless, since germline mosaicism (mutations involved in the germ cells but not identifiable in the blood) has been established in some individuals, healthy parents of a kid with a de novo mutation must be advised that the recurrent chance of affecting the next baby is insignificant[72] (Figure 2)[73].

The pancreatic KATP channels directly regulate insulin release. Multiple subunits of internal rectifying k+ channels 11 (Kir6.2, encoded by KCNJ11) and 4 subunits of the sulfonylurea receptor family (SUR1, encoded by ABCC8) combine to produce the hetero-octamer. GCK phosphorylates glucose to glucose-6-phosphate when it enters the cell, then glycolysis and the Calvin cycle decompose glucose to make ATP. The KATP channel closes due to the elevated ATP/MgATP ratio, depolarizing the cellular membranes and activating voltage-gated Ca^{2+} channels. Insulin is secreted from cells when calcium enters the cells *via* the active voltage-gated calcium channel. A system of transcriptional regulators, including

HNF1A, NEUROD1, HNF4A, PDX1, and HNF1B, modulating the expression of insulin and the growth and division of beta cells. Red labels identify the genes linked to MODY[74,75].

Monogenetic diabetes in children due to mutation in INSs

The INS has been found to have heterogeneous mutations, which may contribute to approximately 10-13 percent of permanent neonatal diabetes occurrences[43,76,77]. Most mutations disrupt the insulin A or B chains. They are projected to prevent cysteine amino acids from forming disulfide bonds with one another by either adding an extra cysteine residue or transforming the existing one. Therefore, INS mutations cause a misfolded proinsulin molecule to be retained and aggregate in the endoplasmic reticulum, which causes the endoplasmic reticulum stress responses to be induced, inhibits protein production, and eventually results in β-cell destruction[78]. Inadequate birth weight, a characteristic of all subcategories of NDM, is the only extrapancreatic symptom present in patients with PNDM and an INS mutation.

Additionally, there is no variation in birth weight between INS mutation carriers and carriers of ABCC8 or activated KCNJ11 mutations. Children reported during the first 6 mo with persistent DM need molecular genetic screening to validate the chromosomal subtype, even though individuals with INS genetic variations are detected later since the range overlaps. Insulin is the sole medication option for individuals with monogenic diabetes because it causes the β-cells to progressively expire[79].

The overwhelming proportion of INS mutant individuals are spontaneous occurrences caused by denovo mutations. About 20% of incidences occur in families with an autosomal dominant transmission pattern. Therefore, 50% of afflicted people can transmit the illness to their offspring[50]. It is worth noting that between 6 and 12 mo, both INS and KCNJI1 mutants are an infrequent cause of irreversible diabetes. When dealing with diabetic newborns, particularly those who lack pancreatic autoantibodies or a high-threat human leukocyte antigens genotype for DM1, this must be considered[80].

Monogenetic diabetes in children due to mutations in the GCK genes

The β-cell's sensor for glucose is the enzyme GCK, which catalyzes the rate-limiting reaction of glucose phosphorylation and allows the cells to react correctly to the level of glycemia[81]. Heterozygous GCK genetic mutations cause familial, moderate, non-progressive hyperglycemia. Nevertheless, the β-cells cannot secrete insulin in response to hyperglycemia if they have homozygous or compound heterozygous abnormalities in both genes that cause complete GCK insufficiency [82-84]. Only 4-5 percent of instances of PNDM are explained by this mechanism. Significant intrauterine developmental impairment and hyperglycemia can be seen as early as the first day of life (birth weight 1700 g). Individuals need to take insulin for a lifetime and don't have any significant additional pancreatic characteristics[85]. The diagnosis must be seriously investigated in consanguineous couples, particularly when both parents show moderate hyperglycemia. Monitoring fasting sugar levels in the parents of each newborn having NDM ought to be mandatory, particularly when there is no known family background of the condition because it is typically asymptomatic. Due to the recessive nature of this kind of diabetes, a patient's future siblings have a 25% chance of developing the condition[86].

Abnormalities in insulin sensitivity and secretion

Most monogenic diabetes in children is caused by gene abnormalities that alter insulin biosynthesis, packing, glucose sensing, or insulin release, resulting in β-cell depletion or malfunction[59,87]. The $CD4^+$ $CD25^+$ regulatory T lymphocytes, wherein overactivation leads to auto-immunity against β-cells, often leading to diabetes in the first three months of life, are the site of other alterations that influence insulin production and are not expressed in pancreatic beta cells. Decreased numbers of β-cells or granules and reduced insulin levels in these globules may be caused by mutations that impair the translational, breakdown, and packaging of insulin. Genes that control glucose sensing are affected by mutations that influence insulin release instead of the formation or degeneration of beta cells. They consist of mitochondrial DNA structural mutations. Most of these abnormalities decrease glucose sensitivity and metabolism, encouraging the open configuration of the K^+ channel and preventing depolarization, which leads to insulin release. Monogenic diabetes, caused by mutations leading to extreme insulin resistance, rarely develops in children. These are primarily brought on by mutations in genes encoding the insulin receptor, which change the gene's biosynthesis and post-translational processing, promote receptor degradation, decrease insulin binding or receptor activation, and more. These result in Leprechauns, Rabson-Mendenhall syndrome, or type A severe insulin resistance. Alternately, hypertriglyceridemia linked to congenital generalized lipoatrophy or familial partial lipodystrophy may cause insulin resistance[88-91].

Miscellaneous monogenetic diabetes in children and infants

The other genetic origins of newborn DM are rare. While evaluating whether to test for additional genetic subtypes, related clinical information and understanding of kinship might be highly significant. About 5 to 10 percent of permanent neonatal diabetes cases are caused by pancreatic hypoplasia or aplasia. Whereas some of these individuals' mutations have already been discovered, the majority of these individuals still lack a genetic diagnosis. There have been two cases of pancreatic agenesis where the transcription factor IPF1 has completely failed due to homozygous or complex heterozygous alterations in the IPF1 gene[92,93]. Since it controls how midgut endodermic stem cells differentiate, IPF1 is crucial for the embryonic maturation of the pancreas. It also plays a role in INS transcription in adulthood. Therefore, IPF1 heterozygous mutations are responsible for a small number of incidences of inherited juvenile-stage diabetes[94]. Additionally, certain polymorphism variations of the gene increase the likelihood of getting type 2 diabetes[95]. Numerous individuals with pancreatic and cerebellar hypoplasia/agenesis from 2 consanguineous families had identical mutations in pancreas transcription factor 1α, which codes for pancreas transcription factor 1-α[96,97].

GLIS3, a transcriptional modulator with high levels of expression, has now been linked to a complicated syndrome that includes gestational hypothyroidism, neonatal diabetes, and dysmorphic traits. Neonatal glaucoma, liver cirrhosis, and

glomerular cysts were also found in some cases. Four probands from three consanguineous families that were not linked to each other had homozygous mutations in the GL1S3 gene so far[98,99].

Multisystemic disorder, immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome manifest in homozygous recessive males with a mutation in the FOXP3 gene[100]. For regulating T cells to mature and perform properly, the genes that encode this protein must be present[101]. Its absence is linked to several autoimmune disorders with early development (enteropathy, DM, eczematous dermatitis, hypothyroidism, cytopenias, *etc*.), which frequently cause the patient to pass away during the initial few years of adulthood. Surprisingly, antibodies against β-cell antigens could be discovered, marking a significant distinction from other PNDM-causing factors. Therapeutic options include bone marrow transplants and immunosuppression. Female heterozygous carriers don't exhibit any symptoms[102].

Below 1% of kids seen in diabetic clinics have syndromic versions of the disease, making them uncommon. Most cases are either incorrectly or never diagnosed due to their rarity and complexity. It is crucial to appropriately diagnose these disorders in children so that difficulties can be anticipated, recognized, and treated. Parents may also choose to receive genetic counseling[103].

The condition known as MIDD is because of an A to G alteration at position m.3243A>G in the mitochondrial DNA that codes for the gene for tRNALeu and is thought to affect up to 1% of diabetics. Beta cell mass reduction, a steady decline in beta cell activity, and a reduction in glucose-induced insulin secretion are assumed to be the effects of mitochondrial malfunction in the extremely metabolically dynamic pancreatic islets. When compared to the percentage of diabetes induced by m.3243A>G, additional mitochondrial DNA genetic variations that have been linked to MIDD are incredibly rare[104,105].

Wolfram syndrome (WFS) is the most prevalent syndromic monogenic diabetes in kids and teenagers. The occurrence of WFS, commonly referred to as diabetes insipidus, DM, optic atrophy, and deafness, is thought to be 1 in 770000. Despite being a nonautoimmune type of diabetes, insulin insufficiency is a common complication in WFS patients due to the selective death of pancreatic beta cells and compromised insulin output. The latest reports link a missense alteration to nonsyndromic, autosomal dominant adult-onset diabetes[106-108].

DIAGNOSIS STRATEGIES OF MONOGENIC DIABETES IN NEONATES AND CHILDREN

Targeted gene sequencing

Targeted therapy is made possible by the earlier diagnosis of monogenic diabetes in neonates and children. Improvements in glycemic control reduce comorbidities from diabetes, and a reduction in the expense and load of medication have all been linked to genetically-targeted therapy[65,109]. According to investigations, monogenic diabetes can be detected by affordable genetic analysis in the right individuals[66,110]. It is critical to differentiate between type 1 and type 2 diabetes and monogenic diabetes to monitor complications, identify extra-pancreatic illnesses that may be present, and identify afflicted and vulnerable members of the family[68,69]. To validate a clinical confirmation of monogenic diabetes, genetic screening must be conducted. Clinicians have various test methods and diagnostic strategies available as the set of genes linked to monogenic types of diabetes rises. Sanger sequencing is still the gold standard for finding single base changes and minor penetrations or removals. Still, it can only diagnose a small number of specific genes and requires previous knowledge of the probably afflicted gene. Carroll and Murphy[75] developed a diagnostic method in which doctors screen the most prevalent types of MODY (GCK, HNF1A, and HNF4A) first and only take into account the less common forms once those three have been ruled out[111].

Whole-exome sequencing (WES)

WES, which focuses primarily on the human genome's protein-coding regions, is a potent method for identifying novel causal genes in monogenic illnesses. WES analysis has recently been a successful strategy for identifying the new genes in MODY-X cases. WES was performed on four Turkish patients from two families who were negative for the most prevalent MODY genes (HNF1A, HNF4A, GCK, and HNF4A). We detected disease-causing missense mutations in novel MODY candidate genes in two families after filtering pathologic variants. Two mutations (p.His307Gln in c-Myc and p.Gly107Ser in ARHGDIA) were not in any database and graded as probably detrimental by functional prediction software, while p.Asp129Asn in CDK4 was previously reported but not in 1000 genome, ESP6500, or ExAc databases[3, 112].

NGS

NGS techniques have replaced Sanger sequencing in most industrial and clinical genomic labs. Several identified genes associated with diabetes can be simultaneously analyzed using next-generation targeted sequencing panels, which are about as expensive as Sanger sequencing to examine a few genes. Most crucially, specialized panels may find mutations in patients who don't have the disease's defining symptoms[113]. The likelihood that variations of ambiguous significance would appear in genetic testing findings is a significant side effect of employing panels. These variations are frequently challenging to interpret regarding illness risk or cause, necessitating additional patient medical data and testing of first-degree relatives to aid the assessment. When it relates to comprehending and explaining data to patients and making clinical care considerations, such situations present a unique difficulty for doctors. Whenever the cause of a variation is unclear, requesting physicians should speak with experts in monogenic diabetes.

THERAPEUTIC OPTIONS FOR THE MANAGEMENT OF MONOGENETIC DIABETES IN CHILDREN

The overall general opinion is that pharmaceutical intervention is not necessary, except for pregnancy, when management is based on fetal genotype, provided that the mild high blood sugar, the absence of long-term abnormalities, and the assessment that management with antidiabetic drugs or the insulin does not affect glycemia[114]. Thirty percent of GCK-MODY participants who received incorrect diagnosis and treatment with glucose-lowering medication, such as insulin, reported hypoglycemia and other negative consequences[115]. Vulnerability to SU is the first therapeutic option in HNF1A-MODY3, a significant and unique distinctiveness of HNF1A-MODY. It has an important implication, especially for individuals misdiagnosed with type 1 diabetes, because they might be able to stop insulin and receive SU medication even after receiving a lot of insulin[116]. Children on oral hypoglycemic drugs or sub-replacement insulin dosages can quit their insulin treatment and switch to low-dose SUs. The smallest quantity of sulfonylurea, such as glyburide (one-half to one 1.25 mg tab), must be used to start them. To get optimal blood glucose control, the dose can be increased. For those using replacement insulin doses, lowering basal insulin by at least 50% and ceasing bolus insulin at the start of SU are recommended. Meglitinides are among the additional therapy choices. In comparison to glibenclamide 1.25 mg, nateglinide 30 mg was demonstrated to produce reduced hypoglycemia in persons with HNF1A-MODY[117].

Repaglinide and nateglinide have been used in a case study of children with HNF1A-MODY. Meglitinides may be the first treatment for kids with HNF1A-MODY instead of SUs, according to this analysis of three teenagers, where the use of the medication was linked to little or infrequent hypoglycemia *vs* persistent hypoglycemia with SUs[118]. Compared to SU, glucagon-like peptide-1 (GLP-1) receptor analogs have been demonstrated to significantly decrease blood sugar concentrations in people with HNF1A-MODY[119]. SUs alone will not provide appropriate blood glucose control in certain people with HNF1A-MODY, or satisfactory control may worsen over time. This appears connected to gaining weight and latency in starting SUs[64]. Although the optimal replacement therapy plan is unknown, alternatives comprise supplementing SUs with metformin, basal insulin, or GLP-1 agonists. A study on the effects of SGLT2 inhibitors in HNF1A-MODY has been published, demonstrating an elevation in glycosuria[120].

Nanotechnology in the diagnosis and treatment of diabetes

The identification of diabetes at an early stage and an assessment of its progression are critical components of diabetic care. Individuals diagnosed with diabetes must consistently check their blood glucose levels to manage and maintain their blood sugar levels effectively, mitigating the risk of developing diabetic complications[121]. The diagnostic tools commonly employed in clinical settings utilize the blood sample obtained by pricking the fingertip with a needle. However, there has been a recent trend towards implementing modern technology for continuous real-time monitoring of blood glucose levels. Glucose sensors are employed to monitor glucose concentrations in either the bloodstream or the interstitial fluid. A glucose sensor typically comprises three essential components: A detector, a transducer, and a reporter. A pressing requirement is to improve glucose sensors to enhance their accuracy and specificity and enable real-time detection[122-124].

The application of nanotechnology has been found to influence glucose sensors significantly. This is primarily due to nanotechnology's ability to enhance the sensors' surface area and improve the electrodes' catalytic activity. Moreover, nanotechnology has also played a crucial role in developing miniaturized nanoscale devices capable of detecting glucose. Recently, surface-enhanced raman spectroscopy-based biosensors have been widely studied to detect diabetes[125]. The utilization of carbon nanotubes (CNTs) has also been explored in the context of glucose detection in urine. The utilization of biopolymer chitosan (CS) aqueous solutions containing dissolved CNTs enables the monitoring of urine glucose levels without any interference[126]. The glucose detection in urine can be facilitated by employing ZnFe2O4 magnetic nanoparticles (NP) (MNPs) with inherent peroxidase-like activity. This research suggests these MNPs can be a colorimetric biosensor[127]. Another study devised a glucometer with flexibility, self-sustainability, and a skin-like appearance. This innovative device was designed to continuously monitor blood glucose levels within the human body, facilitating the proactive management and treatment of diabetes. The functioning mechanism relies on the interplay between piezoelectricity and enzyme processes within arrays of GOx@ZnO nanowires[128] (Figure 3)[129].

Diabetes is a chronic condition with no known cure; nevertheless, it can be effectively managed using many existing medical treatments. The efficacy of the treatment is dependent on the administration of insulin and other pharmacological medicines used to manage diabetes[130]. There is a notable scientific inclination towards advancing non-invasive techniques for administering insulin and/or extending its temporal efficacy through nanotechnology. The delivery of insulin through nanomedicine entails the utilization of polymeric NPs, micelles, metallic NPs, solid lipid nanoparticles, and biodegradable polymer nanoparticles[131]. Polymer-based delivery approaches commonly incorporate polyethylene glycol (PEG), wherein peptide or protein medications such as insulin are conjugated with PEG to enhance solubility, permeability, and stability during oral administration. Likewise, there have been notable advancements in the utilization of insulin *via* the oral route with the application of micellar formulations[132] (Figure 4)[133].

Liposomes are considered to be more appropriate and enduring structures compared to micelles. Consequently, certain variations of liposomes have been created and examined in animal models to assess their efficacy in delivering insulin. The oral administration of liposomal insulin has demonstrated enhanced bioavailability compared to the free version [134]. Nanoparticles loaded with insulin have been created utilizing a range of polymers such as CS, polylactide-co-glycolic acid, and dextran. The utilization of solid lipid nanoparticles has been explored to deliver insulin[135].

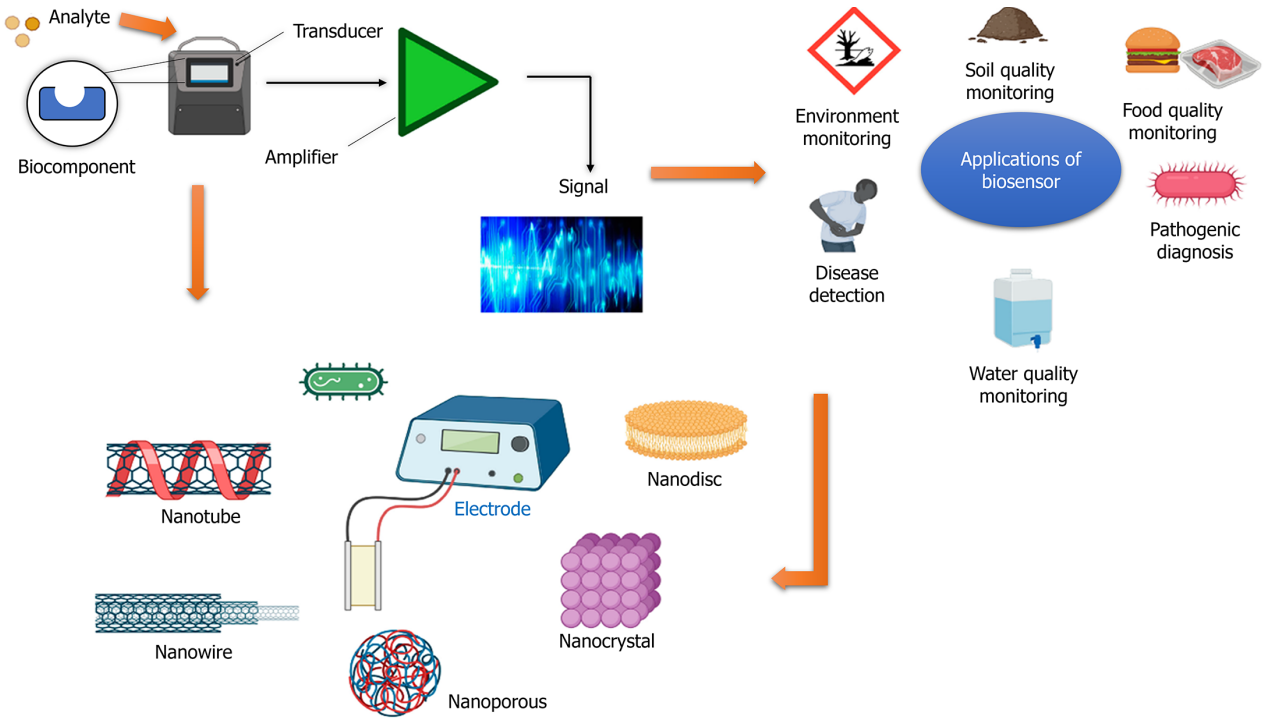

Figure 3 The utilization of a glucose biosensor in advanced biotechnology and research applications. Citation: Shoaib A, Darraj A, Khan ME, Azmi L, Alalwan A, Alamri O, Tabish M, Khan AU. A Nanotechnology-Based Approach to Biosensor Application in Current Diabetes Management Practices. Nanomaterials (Basel) 2023; 13. Copyright ©The MDPI AG. Published by MDPI AG.

FUTURE DIRECTIONS AND RESEARCH

The investigation of genetic aspects of childhood monogenic diabetes not only provides valuable insights into the existing body of knowledge but also establishes a foundation for promising future avenues of research. With the progression of genetic analysis, there is an increasing potential to discover new gene mutations and comprehend their complex involvement in the development of monogenic diabetes. This can potentially reveal previously unknown disease subtypes and enhance our comprehension of the underlying mechanisms[136]. Furthermore, incorporating genomic data in conjunction with other 'omics' fields, like transcriptomics and metabolomics, can offer a comprehensive understanding of the molecular landscape of the disease. The construction of comprehensive databases through collaborative efforts in data sharing and multinational consortia can significantly assist clinicians in accurately diagnosing patients and selecting appropriate treatment options[137]. In addition, the prospect of gene treatments and precision medicine strategies presents a promising perspective, wherein customized interventions aimed at specific genetic abnormalities have the potential to profoundly transform the treatment of pediatric monogenic diabetes[138]. In essence, comprehending and effectively handling monogenic diabetes is closely linked to the ever-evolving field of genetics. It offers the potential for groundbreaking progress that will significantly impact the provision of diabetes care for children.

CONCLUSION

As a result of the challenges associated with identifying monogenic forms of diabetes in pediatrics, there is an increasing tendency for these conditions to be underdiagnosed, thereby overlooking potential opportunities for treatment strategies based on genetic factors. The misdiagnosis of diabetes can be attributed to several factors, including the clinical and hereditary variability of its subtypes, the complex relationship between clinical and polygenic types, the high cost of genetic screening, lack of healthcare insurance coverage, and limited knowledge of the condition among medical professionals. Integrating biomarkers with phenotype is a promising approach that can potentially speed up and improve the accuracy of genetic diagnoses. The clinical implications of this discovery for both the patient and their family, notwithstanding the relatively low prevalence of monogenic forms of diabetes, support the appropriate utilization of genetic testing. Assessing an inherited genetic form of diabetes necessitates specific consideration of several factors, including the absence of typical symptoms associated with type 1 or type 2 diabetes, early onset of the condition, familial predisposition, and extrapancreatic abnormalities. The prognosis and management of monogenic diabetes in pediatric and adolescent populations can be improved by expanding knowledge regarding the condition and facilitating a more approachable assessment process.

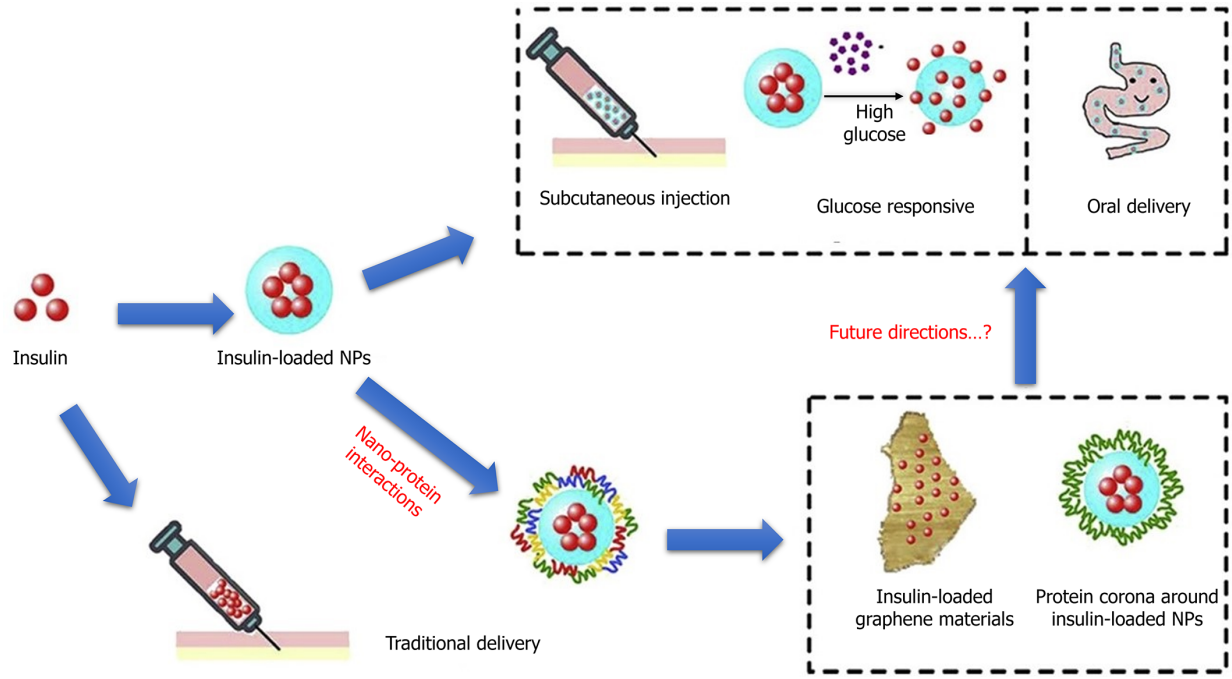

Figure 4 Interactions between proteins and nanomaterials, such as those between proteins and insulin-loaded nanoparticles. Citation: Zhang T, Tang JZ, Fei X, Li Y, Song Y, Qian Z, Peng Q. Can nanoparticles and nano-protein interactions bring a bright future for insulin delivery? Acta Pharm Sin B 2021; 11: 651-667. Copyright ©The Amsterdam: Elsevier. Published by Amsterdam: Elsevier.

FOOTNOTES

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ORIGINAL ARTICLE

Case Control Study

Comparative analysis of Nε-carboxymethyl-lysine and inflammatory markers in diabetic and non-diabetic coronary artery disease patients

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Abstract

BACKGROUND

Coronary artery disease (CAD) is a major cause of death worldwide, and India contributes to about one-fifth of total CAD deaths. The development of CAD has been linked to the accumulation of Nε-carboxymethyl-lysine (CML) in heart muscle, which correlates with fibrosis.

AIM

To assess the impact of CML and inflammatory markers on the biochemical and cardiovascular characteristics of CAD patients with and without diabetes.

METHODS

We enrolled 200 consecutive CAD patients who were undergoing coronary angiography and categorized them into two groups based on their serum glycosylated hemoglobin (HbA1c) levels (group I: HbA1c ≥ 6.5; group II: HbA1c < 6.5). We analyzed the levels of lipoproteins, plasma HbA1c levels, CML, interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and nitric oxide.

RESULTS

Group I (81 males and 19 females) patients had a mean age of 54.2 ± 10.2 years, with a mean diabetes duration of 4.9 ± 2.2 years. Group II (89 males and 11 females) patients had a mean age of 53.2 ± 10.3 years. Group I had more severe CAD, with a higher percentage of patients with single vessel disease and greater stenosis severity in the left anterior descending coronary artery compared to group II. Group I also exhibited a larger left atrium diameter. Group I patients exhibited significantly higher levels of CML, TNF-α, and IL-6 and lower levels of nitric oxide as compared with group II patients. Additionally, CML showed a significant positive correlation with IL-6 (r = 0.596, P = 0.001) and TNF-α (r = 0.337, P = 0.001) and a negative correlation with nitric oxide (r=-4.16, P = 0.001). Odds ratio analysis revealed that patients with CML in the third quartile (264.43-364.31 ng/mL) were significantly associated with diabetic CAD at unadjusted and adjusted levels with covariates.

CONCLUSION

CML and inflammatory markers may play a significant role in the development of CAD, particularly in diabetic individuals, and may serve as potential biomarkers for the prediction of CAD in both diabetic and non-diabetic patients.

Key Words: Coronary artery disease; Diabetes; Nε-carboxymethyl-lysine; Inflammatory markers; Interleukin-6; Tumor necrosis factor alpha; Nitric oxide

Core Tip: Coronary artery disease (CAD) incidence is substantial in India.Its development is linked to the accumulation of Nε-carboxymethyl-lysine (CML). We assessed the impact of CML and inflammatory markers on biochemical and cardiovascular characteristics in diabetic and non-diabetic CAD patients. Diabetic patients exhibited elevated CML, tumor necrosis factor alpha, and interleukin 6 levels with reduced nitric oxide levels. CML levels displayed a significant correlation with interleukin 6, tumor necrosis factor alpha, and nitric oxide. The third quartile of CML was associated with diabetic CAD, suggesting its role as a biomarker in CAD prediction for diabetic and non-diabetic patients.

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INTRODUCTION

Heart disease, specifically heart failure (HF) and coronary artery disease (CAD), is a major contributor to mortality in both developed and developing countries[1]. The World Health Organization states that the most common cause of death is cardiovascular disease (CVD), resulting in 17.9 million annual deaths. Subsequently, cancer, chronic respiratory ailments, and diabetes trail behind as causes of mortality[2]. In diabetic individuals with CAD, inadequate management of blood sugar levels is linked to both hospitalization and mortality[3]. Diabetes mellitus is a major risk factor for the cause and progression of atherosclerosis[4,5].

Some recent literature evidence suggests that advanced glycation end products (AGEs) play an important role in the acceleration of vascular disease[6]. AGEs are formed from the non-enzymatic reaction of sugars and proteins, leading to oxidative stress, inflammation, and endothelial dysfunction through various mechanisms[7]. In hyperglycemia, the accumulation of AGEs is thought to play a role in the onset of diabetic complications. AGE buildup can modify tissue structure, affecting its properties and making it more resistant to breaking down[8]. One of the major AGEs, Nε-carboxymethyl-lysine (CML) is formed by the non-enzymatic glycation and oxidation of monosaccharides (glucose) and proteins (lysine). The attachment of AGEs to receptor for AGEs (RAGE)may result in impaired cellular communication, protein structure and functional alterations, and mitochondrial malfunction, ultimately resulting in cellular demise. RAGE binding can also increase reactive oxygen species and stimulate inflammatory signaling through tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). It also affects endothelial function by altering nitric oxide levels[9].

Subsequently, new evidence suggested that CML has made a major contribution to the development of CAD[10]. CML found in heart muscle shows a positive correlation with fibrosis and cardiac disease[11]and promotes hypertrophy, apoptosis, and myocardial fibrosis[12]. Elevated CML levels have been linked to poor collateralization in chronic total occlusion in diabetic CAD patients[13]. Along with CAD, CML is also significantly associated with many other diseases, like diabetic retinopathy, diabetic nephropathy, diabetic neuropathy, and cancer[14].

In this study, we assessed the impact of CML in association with inflammatory markers on biochemical and cardiovascular characteristics in diabetic and non-diabetic CAD patients. We aimed to gain new insights while exploring the relationship between diabetes and CAD, which may open future prospects for therapeutic intervention in such patients.

MATERIALS AND METHODS

Study population

This cross-sectional study was conducted at the Department of Biochemistry, G.B. Pant Institute of Postgraduate Medical Education and Research (GIPMER), New Delhi, India. We enrolled 200 age-matched and sex-matched, angiography-confirmed patients diagnosed with CAD from both OPD & IPD of Department of Cardiology. All patients signed an informed consent. The study was conducted in accordance with internationally accepted recommendations for clinical investigation (the Declaration of Helsinki of the World Medical Association, revised October 2013) with approval from the ethics committee of Maulana Azad Medical College and associated hospitals, New Delhi, India.

Sample collection

Venous blood (5 mL) was drawn under aseptic conditions from consented patients. Further, a 3-mL sample was transferred to an EDTA vial for glycosylated hemoglobin (HbA1c) and special chemistry analysis, and the remaining sample was transferred to a glucose vial for blood sugar analysis. Patients with HbA1c level ≥6.5% or having a previous diagnosis of diabetes were considered as diabetic CAD (group I), while patients with level <6.5% were categorized as non-diabetic CAD (group II). Group II patients with no prior history of diabetes and no history of anti-diabetic medication were classified as non-diabetic CAD. The serum levels of HbA1C were measured by a fully automated analyzer, whereas the CML, IL-6, TNF-α, and nitric oxide levels were determined by enzyme-linked immunosorbent assay methods.

Clinical assessment

Independent senior cardiologists utilized the angiographic data from the catheterization laboratory to calculate the severity of CAD using the Gensini scoring (GS) system. The left coronary artery was separated into left anterior descending (LAD), circumflex, and obtuse marginal branches, while the right coronary artery (RCA) was considered a single artery. The lesion score for each coronary segment was multiplied by a location-based factor, and then the scores were added together to calculate the GS.

GS system

The GS was determined by adding the scores from each coronary segment as follows: one point for 25% stenosis; two points for 26%-50% stenosis; four points for 51%-75% stenosis; eight points for 76%-90% stenosis; sixteen points for 91%-99% stenosis; and 32 points for total occlusion. The significance of the location of the lesion in the coronary circulation was also considered, with 5 points for the left main coronary artery, 2.5 points for the proximal LAD coronary artery and proximal left circumflex artery, 1.5 points for the mid-LAD coronary artery, 1 point for the RCA, the distal segment of the LAD coronary artery, the posterolateral artery, and the obtuse marginal artery, and 0.5 points for other segments[15].

Doppler echocardiography examination

A standard two-dimensional, M-mode, and Doppler echocardiography examination was conducted using the Philips EpiQ-7C echocardiography system. The examination measured various parameters including the dimension of the left atrium (LA) and the aortic root. The left ventricular ejection fraction (LVEF) was also calculated using Simpson's method [16].

Cardiovascular risk factor assessment

Patients over the age of 18 years who were confirmed with the diagnosis of CAD by resting electrocardiogram or coronary angiography with >50% stenosis were included in this study. Blood pressure was measured as an average of two readings recorded at least 5 min apart while the participants rested in a seated position. Hypertension was identified when the subject was either having a history of hypertension or a systolic blood pressure of ≥140 mmHg or a diastolic blood pressure of ≥90 mmHg. Patients with total cholesterol (TC) (>200 mg/dL), triglycerides (>150 mg/dL), high-density lipoprotein cholesterol (HDL-C) (<40 mg/dL), or low-density lipoprotein cholesterol (LDL-C) (>100 mg/dL) were defined as having dyslipidemia. Additionally, patients with renal or hepatic impairment as well as those who had undergone previous therapies such as coronary artery bypass graft surgery or percutaneous coronary intervention were excluded from the study.

Statistical analysis

The SPSS version 21 (IBM Corp., Chicago, IL, United States) was used to analyze the data. The mean and standard deviation and frequency and percentage were used to express quantitative and qualitative data, respectively. For quantitative data, an independent *t*-test was performed to compare two independent variables. The normality of the data was checked by the Kolmogorov-Smirnov test. Student's *t*-test, analysis of variance, and Mann-Whitney *U* test were used to compare parametric and non-parametric variables. All statistical tests were carried out at a $P < 0.05$ significance level.

Table 1 Demographic characteristics in diabetic coronary artery disease patients and non-diabetic coronary artery disease patients

Parameter	Group I, *n* = 100	Group II, *n* = 100	*P* value[1]
Age	54.2 ± 10.2	53.2 ± 10.3	0.473[2]
Male:Female	81 (81%): 19 (19%)	89 (89%): 11 (11%)	0.82[3]
Non-vegetarian diet	80%	60%	0.001[3]
Smoker	65%	50%	0.022[3]
Alcohol consumption	26%	25%	0.500[3]
Tobacco chewer	49%	39%	0.100[3]
Hypertensive	39%	20%	0.001[3]
Systolic blood pressure in mmHg as median	125.5 (118.0-140.0)	120.0 (114.0-129.5)	0.001[4]
Diastolic blood pressure in mmHg as median	80.0 (72.0-84.0)	80.0 (70.0-80.0)	0.089[4]
Medications			
Statin	79%	89%	0.041[3]
Beta-blocker	58%	73%	0.018[3]
ACE inhibitor	17%	5%	0.005[3]

[1]*P* value < 0.05 is considered significant.
[2]Student's *t* test.
[3]$\chi 2$ test.
[4]Mann Whitney *U* test. Group I: Diabetic coronary artery disease patients.
Group II: Non-diabetic coronary artery disease patients. ACE: Angiotensin converting enzyme.

RESULTS

Demographic characteristics

The mean age of group I was 54.2 ± 10.2 years, while the mean age for group II was 53.2 ± 10.3 years (P = 0.473). There was a male sex predominance with males constituting 81% in group I and 89% in group II. In group I, the duration of diabetes was 4.9 ± 2.2 years. Hypertension was more prevalent in group I (39%) than in group II (20%) (P = 0.001). The median systolic blood pressure was significantly higher in group I [125.50 mmHg; 95% confidence interval (CI): 118.0-140.0] compared to group II (120 mmHg; 95%CI: 114.0-129.5) (P = 0.001). In relation to medications, statin use was 79% in group I and 89% in group II. Beta-blockers were taken by 53 (53%) subjects in group I and 73 (73%) subjects in group II. Only 5 (5%) subjects in group II, compared to 17 (17%) in group I, were taking angiotensin converting enzyme (ACE) inhibitors (Table 1). The ACE inhibitor usage was lower as the drug history was taken just before the cardiac catheterization. Subsequently, patients were started on an ACE inhibitor once they were stable.

Cardiovascular characteristics

Group I consisted of 57 patients with single vessel disease (SVD), 27 patients with double vessel disease, and 8 patients with triple vessel disease. However, group II had 35 patients with SVD, 36 patients with double vessel disease, and 11 patients with triple vessel disease. Eight patients in group I and fourteen patients in group II had normal angiograms (P = 0.016).

The mean and standard deviation of severity of stenosis in the LAD artery were observed as 90.51% ± 8.51%, in the left circumflex (LCX) artery as 90.91% ± 8.80%, and in the RCA as 90.32% ± 10.15% in group I. On the other hand, in group II, the mean and standard deviation of stenosis in the LAD were 87.85% ± 12.31%, in the LCX were 82.22% ± 22.33%, and in the RCA were 89.26% ± 12.90%. The GS was higher in group I, with a score of 26 (12–44) compared with group II with a score of 20 (12-40). Group I had a larger LA diameter of 2.93 ± 0.32 cm compared to 2.83 ± 0.39 cm in group II (P = 0.04). The aortic root diameter was slightly larger in group I at 2.15 ± 0.39 mm compared to 2.10 ± 0.40 mm in group II. Further, group I had a mean LVEF of 45.60% ± 12.04%, and group II had a mean EF of 46.70% ± 12.01%.

The patients were categorized based on their LVEF in Table 2. In group I, 38% of patients had preserved EF (LVEF ≥ 50%), 13% had mild EF reduction (LVEF 41%-49%), and 49% had reduced EF (LVEF < 40%). In group II, 43% of patients had preserved EF, 14% had mild EF reduction, and 43% had reduced EF. Anterior wall myocardial infarction was experienced by 39% of patients in group I and 39% of patients in group II, and inferior wall myocardial infarction was experienced by 26% of patients in group I and 21% of patients in group II.

Comparison of biochemical parameters in group I and group II

The TC, triglycerides levels, and very-LDL levels were found to be significantly higher in group I compared to group II (P = 0.006, P = 0.001, and P = 0.001, respectively). Further, both HbA1c and the blood sugar levels were found to be

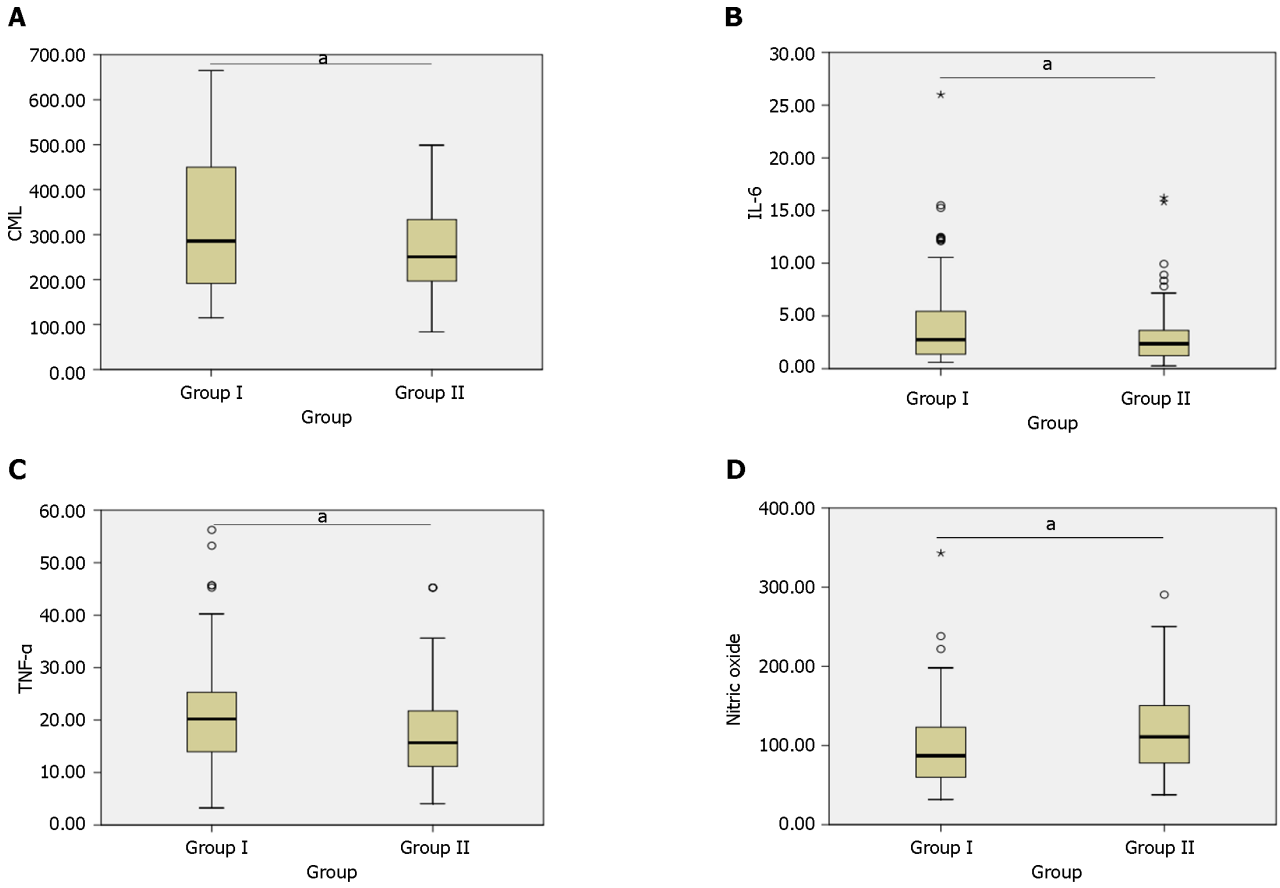

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Figure 1 Comparison of serum Nε-carboxymethyl-lysine, interleukin 6, tumor necrosis factor alpha, and nitric oxide between group I and II. A: Serum Nε-carboxymethyl-lysine (CML) level; B: Serum interleukin-6 (IL-6) level; C: Serum tumor necrosis factor-alpha (TNF-α) level; D: Serum nitric oxide. [a]Biochemical markers CML, IL-6, TNF-α, and nitric oxide showed a difference between diabetic coronary artery disease patients and non-diabetic coronary artery disease patients. *Significant difference of biochemical markers between Group I: Diabetic coronary artery disease patients; and Group II: Non-diabetic coronary artery disease patients.

significantly higher in group I compared to group II (P = 0.001). The abovementioned intergroup comparison between biochemical parameters has been shown in Table 3.

Association of CML, IL-6, TNF-α, and nitric oxide between group I and group II

The comparison of CML, IL-6, TNF-α, and nitric oxide between group I and group II (Figure 1) showed significant differences between the two groups: serum CML (264.43, 95%CI: 193.19-364.34 *vs* 250.68, 95%CI:195.95-333.70, P = 0.031), IL-6 (2.75, 95%CI: 1.36-5.50 *vs* 2.36, 95%CI: 1.23-3.60, P = 0.011), TNF-α (20.20, 95%CI: 13.65-25.32 *vs* 15.67, 95%CI: 11.14-21.79, P = 0.006), and nitric oxide (87.09, 95%CI: 59.84-124.37 *vs* 110.86, 95%CI: 77.00-150.00, P = 0.002).

Association of lipid parameters between group I and group II

Table 4 shows the lipid profile of individuals in group I and group II. In group I, 17% of individuals had high TC levels (> 200 mg/dL), whereas group II had a lower proportion of individuals with high TC levels (8%). The difference between the groups was significant with a P value of 0.043. In group I, 49% had high triglycerides levels (> 150 mg/dL), while 51% had normal levels (< 150 mg/dL). In group II, a significantly lower proportion of individuals had high triglyceride levels (24%), and a significantly higher proportion had normal levels (76%), with a P value of 0.001. A higher proportion of individuals in group I had low levels (< 40 mg/dL) of HDL (86%) compared to those with normal levels (> 40 mg/dL) (14%). In contrast, group II had a lower proportion of individuals with low HDL levels (73%) and a higher proportion with normal levels (27%) (P = 0.017). In group I, 70% of patients had normal LDL-C levels (< 100 mg/dL), while 30% had high levels (> 100 mg/dL). In group II, 80% of individuals had normal LDL-C levels and 20% had high levels, P = 0.094.

Correlation and logistic regression analysis between CML, inflammatory markers, and lipid parameters

In the correlation analysis, CML exhibited significant positive correlations with IL-6 (r = 0.596), TNF-α (r = 0.337), TC(r = 0.21), HbA1c (r = 0.14), and the GS (r = 0.19) in the combined data from both group I and group II. The correlations of CML (group I *vs* group II), IL-6 (r = 0.502 *vs* r = 0.673), TNF-α (r = 0.256 *vs* r = 0.436), and nitric oxide (r = -0.484 *vs* r = -0.283) between the two groups were significant (Table 5). The linear regression analysis of CML revealed significant positive associations with IL-6 (r^2 = 0.181, P = 0.001), TNF-α (r^2 = 0.142, P = 0.001), TC (r^2 = 0.056, P = 0.001), HbA1c (r^2 =

Table 2 Cardiovascular characteristics in diabetic coronary artery disease patients and non-diabetic coronary artery disease patients

Parameter	Group I, *n* = 100	Group II, *n* = 100	*P* value[1]
Angiography findings			
Single vessel disease	57 (57%)	35 (35%)	0.016[2]
Double vessel disease	27 (27%)	36 (36%)	
Triple vessel disease	8 (8%)	15 (15%)	
Normal angiogram	8 (8%)	14 (14%)	
Stenosis in LAD as %	90.51 ± 8.51	87.85 ± 12.31	0.05[3]
Stenosis in LCX as %	90.91 ± 8.80	82.22 ± 22.33	0.23[3]
Stenosis in RCA as %	90.32 ± 10.15	89.26 ± 12.90	0.73[3]
Gensini score	26 (12-44)	20 (12-40)	0.47[3]
2D echocardiography parameters			
Left atrium diameter in cm	2.93 ± 0.32	2.83 ± 0.39	0.01[3]
Aortic root diameter in mm	2.15 ± 0.39	2.10 ± 0.40	0.27[3]
LVEF	45.60 ± 12.04	46.70 ± 12.01	0.49[3]
Preserved ejection fraction, LVEF ≥ 50%	38 (38%)	43 (43%)	0.69[2]
Mild ejection fraction, LVEF 41%-49%	13 (13%)	14 (14%)	
Reduced ejection fraction (LVEF < 40%)	49 (49%)	43 (43%)	
AWMI	39 (39%)	39 (39%)	0.56[2]
IWMI	26 (26%)	21 (21%)	

[1]*P* value < 0.05 is considered significant.
[2]$\chi 2$ test.
[3]Mann Whitney *U* test.
Group I: Diabetic coronary artery disease patients; Group II: Non-diabetic coronary artery disease patients. AWMI: Anterior wall myocardial infarction; IWMI: Inferior wall myocardial infarction; LAD: Left anterior descending; LCX: Left circumflex; LVEF: Left ventricular ejection fraction; RCA: Right coronary artery.

0.057, $P = 0.001$), and the GS ($r^2 = 0.027$, $P = 0.02$). Additionally, CML showed a significant negative association with nitric oxide ($r^2 = 0.163$, $P = 0.001$) (Figure 2).

The association between quartiles of CML and diabetic CAD was revealed by logistic regression analysis, while accounting for various covariates in separate models (Table 6). The first quartile of CML (83.73-193.18 ng/mL) served as the reference category. In the unadjusted model, the third quartile (264.43-364.31 ng/mL) had an odds ratio of 2.12 (95%CI: 1.17-3.85, $P < 0.01$). Following adjustments for non-vegetarian diet and hypertension (model 2), the odds ratio for the third quartile rose to 3.05 (95%CI: 1.31-7.06, $P = 0.01$). Furthermore, upon introducing further adjustments in Model 3, encompassing TC, triglycerides, LDL-C, IL-6, and TNF-α, the odds ratio for the third quartile became 3.32 (1.30-8.44, $P = 0.01$) while retaining its statistical significance.

DISCUSSION

CML is an AGE involved in the pathogenesis of CVD[17]. Recent studies have demonstrated that CML is linked to endothelial and cardiac dysfunction, left ventricular diastolic dysfunction, and an increase in carotid intima-media thickness, which is a subclinical marker of atherosclerosis in patients with type 2 diabetes[18]. In our cross-sectional study, we found an association between CML, inflammatory markers, and nitric oxide in both diabetic and non-diabetic CAD patients.

In our study, we observed that group I had a significantly higher frequency of risk factors including non-vegetarian diet intake, smoking, and hypertension. Further, we observed that group I had a higher number of individuals with SVD and a greater severity of stenosis in the LAD and LCX coronary arteries. However, in non-diabetic patients, the LAD was found to be the most affected[19]. Further, we observed that in group I, the diameter of the LA was significantly higher suggesting the chronicity of the disease. The incidence of anterior wall myocardial infarction was similar in both groups; the frequency of inferior wall myocardial infarction was higher in group I than in group II. The LVEF was decreased in both the groups. It has been reported previously that lower LVEF is common in diabetic CAD patients[20].

Table 3 Biochemical parameters in diabetic coronary artery disease patients and non-diabetic coronary artery disease patients

Biochemical parameters	Group I, median (25%-75% quartile)	Group II, median (25%-75% quartile)	*P* value[1]
Total cholesterol in mg/dL	143.50 (118.00-183.50)	132.00 (100.25-163.75)	0.006[2]
Triglycerides in mg/dL	150.00 (106.25-214.00)	114.00 (75.00-148.75)	0.001[2]
HDL-C in mg/dL	33.40 (27.33-38.98)	34.55 (28.70-41.00)	0.449[2]
LDL-C in mg/dL	78.00 (53.50-108.80)	73.00 (52.00-92.75)	0.278[2]
VLDL-C in mg/dL	29.00 (20.85-42.00)	23.00 (15.00-30.00)	0.001[2]
Random blood sugar in mg/dL	213.00 (131.50-275.75)	113.00 (99.00-135.00)	0.001[2]
HbA1c as %	8.09 (7.10-10.20)	5.70 (5.40-5.98)	0.001[2]
Urea in mg/dL	29.00 (23.00-39.60)	28.55 (24.85-34.00)	0.177[2]
Creatinine in mg/dL	0.90 (0.80-1.20)	1.00 (0.80-1.10)	0.811[2]
Total bilirubin in mg/dL	0.40 (0.30-0.70)	0.50 (0.40-0.69)	0.260[2]
Total protein in gm/dL	7.10 (6.80-7.60)	7.10 (6.73-7.48)	0.441[2]
Albumin in gm/dL	4.20 (4.00-4.40)	4.28 (4.00-4.50)	0.281[2]
ALP in U/L	108 (87.00-133.00)	95.50 (84.25-110.00)	0.054[2]
SGOT in U/L	26 (21.00-45.00)	30.00 (22.00-47.50)	0.240[2]
SGPT in U/L	28 (20.00-43.00)	29.95 (22.00-49.00)	0.187[2]
Sodium in mEq/L	136.00 (134.00-139.00)	139.00 (136.00-141.00)	0.001[2]
Potassium in mEq/L	4.60 (4.30-4.90)	4.35 (4.10-4.80)	0.002[2]
CML in ng/mL	264.43 (193.19- 364.34)	250.68 (195.95-333.70)	0.031[2]
IL-6 in pg/mL	2.75 (1.36-5.50)	2.36 (1.23-3.60)	0.011[2]
TNF-α in pg/mL	20.2 (13.65-25.32)	15.67 (11.137-21.785)	0.006[2]
Nitric oxide in nmol/mL	87.09 (59.84-124.37)	110.86 (77.00-150.00)	0.002[2]

[1]*P* value < 0.05 is considered significant.
[2]Mann Whitney *U* test.
Group I: Diabetic coronary artery disease patients; Group II: Non-diabetic coronary artery disease patients. ALP: Alkaline phosphatase; CML: Nε-carboxymethyl-lysine; HbA1c: Glycosylated hemoglobin; HDL-C: High-density lipoprotein cholesterol; IL-6: Interleukin-6; LDL-C: Low-density lipoprotein cholesterol; SGOT: Glutamic-oxalacetic transaminase; SGPT: Glutamic-pyruvic transaminase; TNF-α: Tumor necrosis factor-alpha; VLDL-C: Very low-density lipoprotein cholesterol.

Table 4 Percentage of dyslipidemia in diabetic coronary artery disease patients and non-diabetic coronary artery disease patients

Parameter	Group I	Group II	*P* value[1]
High total cholesterol, > 200 mg/dL	17 (17%)	8 (8%)	0.043
Normal total cholesterol, < 200 mg/dL	83 (83%)	92 (92%)	
High triglycerides, > 150 mg/dL	49 (49%)	24 (24%)	0.001
Normal triglycerides, < 150 mg/dL	51 (51%)	76 (76%)	
Low HDL-C, < 40 mg/dL	86 (86%)	73 (73%)	0.017
Normal HDL-C, > 40 mg/dL	14 (14%)	27 (27%)	
High LDL-C, > 100 mg/dL	70 (70%)	80 (80%)	0.094
Normal LDL-C, < 100 mg/dL	30 (30%)	20 (20%)	

[1]*P* value < 0.05 is considered significant. Group I: Diabetic coronary artery disease patients; Group II: Non-diabetic coronary artery disease patients. HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

Table 5 Correlation analysis of Nε-carboxymethyl-lysine with inflammatory markers, nitric oxide, Gensini score, and biochemical parameters

Parameter	Subcategory	Combined (group I and group II)		Group I		Group II	
		Correlation coefficient	*P* value	Correlation coefficient	*P* value	Correlation coefficient	*P* value
CML	Gensini score	0.193	0.006	0.056	0.577	0.353	0.001
	IL-6	0.596	0.001	0.502	0.001	0.673	0.001
	TNF-α	0.337	0.001	0.256	0.01	0.436	0.001
	Nitric oxide	-0.416	0.001	-0.484	0.001	-0.283	0.004
	TC	0.216	0.002	0.25	0.01	0.109	0.281
	Triglycerides	0.156	0.027	0.087	0.389	0.169	0.093
	HDL-C	-0.064	0.372	-0.105	0.298	0.006	0.953
	LDL-C	0.251	0.001	0.289	0.003	0.151	0.134
	VLDL-C	0.131	0.065	0.045	0.654	0.176	0.081
	Random blood sugar	-0.011	0.875	-0.204	0.052	0.081	0.43
	HbA1c	0.14	0.048	0.006	0.951	0.044	0.66
	Urea	-0.004	0.953	-0.006	0.957	-0.046	0.653
	Creatinine	0.059	0.405	0.129	0.202	-0.047	0.646
	Total bilirubin	0.053	0.458	0.083	0.413	0.08	0.428
	Total protein	0.086	0.229	0.183	0.07	-0.055	0.585
	Albumin	0.062	0.387	0.201	0.046	-0.064	0.525
	Alkaline phosphatase	0.042	0.556	0.003	0.975	0.033	0.743
	SGOT	-0.061	0.395	-0.056	0.581	-0.032	0.754
	SGPT	0.019	0.793	0.102	0.317	-0.027	0.788
	Sodium	0.022	0.762	0.059	0.559	0.079	0.432
	Potassium	0.116	0.103	0.076	0.452	0.102	0.313
	Duration of diabetes	-	-	0.494	0.001	-	-

Group I: Diabetic coronary artery disease patients; Group II: Non-diabetic coronary artery disease patients. CML: Nε-carboxymethyl-lysine; HbA1c: Glycosylated hemoglobin; HDL-C: High-density lipoprotein cholesterol; IL-6: Interleukin 6; LDL-C: Low-density lipoprotein cholesterol; SGOT: Glutamic-oxalacetic transaminase; SGPT: Glutamic-pyruvic transaminase; TC: Total cholesterol; TNF-α: Tumor necrosis factor-alpha; VLDL-C: Very low-density lipoprotein cholesterol.

In the comparison of the biochemical profile, our study found that diabetic CAD patients exhibited significantly higher levels of TC, triglycerides, very-LDL, HbA1c, and potassium levels as well as significantly lower levels of HDL-C and serum sodium compared to non-diabetic CAD patients (Tables 2 and 3). Additionally, we observed that the serum levels of CML, TNF-α, and IL-6 were significantly higher, while the serum levels of nitric oxide were significantly lower in diabetic CAD patients. Similarly, Banach *et al*[21] suggested that dyslipidemia is a common occurrence among diabetic CAD patients and that individualized lipid-lowering therapy can effectively reduce associated complications and risks. Zhao *et al*[22](2023) suggested that patients with acute decompensated HF who had potassium levels outside the range of 3.50 to 4.00 mmol/L, lower levels of sodium, and hypochloremia had a worse short-term prognosis. There was also a positive correlation between the number of electrolyte imbalances and an adverse short-term prognosis among these patients[22]. Similarly, Ahmed *et al*[23] found that elevated CML levels have been linked to the development of ischemic heart disease in patients with type 2 diabetes. Koshino *et al*[24] suggested that increased levels of inflammatory markers (IL-6 and TNF-α) from their baseline increase the risk of CVD and are associated with long-term cardiovascular mortality and cardiovascular death. Similarly, Adela *et al*[25] found lower nitric oxide levels in subjects suffering from diabetes for more than 5 years.

Further, in the correlation analysis (Table 5), CML was overall positively correlated with the GS, IL-6, TNF-α, TC, LDL-C, and HbA1c and negatively correlated with nitric oxide and HDL-C. In group I, CML showed a positive correlation with IL-6, TNF-α, TC, and LDL-C, and a negative correlation with nitric oxide. Furthermore, in group II, CML showed a positive correlation with the GS, IL-6, and TNF-α and a negative correlation with nitric oxide.

Table 6 Logistic regression analysis of Nε-carboxymethyl-lysine for risk of diabetic coronary artery disease

Risk model	CML quartile (range) (group I, *n*; group II, *n*)	Exp (B)	95%CI (lower-upper)	Significance
Model 1: Unadjusted	CML first quartile (83.73-193.18 ng/mL) (group I, *n* =27; group II, *n* =23)	Ref	Ref	Ref
	CML second quartile (193.19-264.42 ng/mL) (group I, *n* = 16; group II, *n* = 34)	0.85	0.48-1.48	0.57
	CML third quartile (264.43-364.31 ng/mL) (group I, *n* = 23; group II, *n* = 27)	2.12	1.17-3.85	0.01
	CML fourth quartile (364.32-665.00 ng/mL) (group I, *n* = 34; group II, *n* = 16)	1.17	0.67-2.04	0.57
Model 2: Model 1 + age + sex + non-vegetarian diet + hypertension	CML first quartile (83.73-193.18 ng/mL)	Ref	Ref	Ref
	CML second quartile (193.19-264.42 ng/mL)	0.57	0.27-1.23	0.15
	CML third quartile (264.43-364.31 ng/mL)	3.05	1.31-7.06	0.01
	CML fourth quartile (364.32-665.00 ng/mL)	1.81	0.82-3.99	0.13
Model 3: Model 2+ total cholesterol + triglycerides + LDL-C + IL-6 + TNF-α	CML first quartile (83.73-193.18 ng/mL)	Ref	Ref	Ref
	CML second quartile (193.19-264.42 ng/mL)	0.84	0.35-2.02	0.70
	CML third quartile (264.43-364.31 ng/mL)	3.32	1.30-8.44	0.01
	CML fourth quartile (364.32-665.00 ng/mL)	2.49	1.03-6.04	0.04

Group I: Diabetic coronary artery disease patients; Group II: Non-diabetic coronary artery disease patients. CI: Confidence interval; CML: Nε-carboxymethyl-lysine; IL-6: Interleukin 6; LDL-C: Low-density lipoprotein; TNF-α: Tumor necrosis factor-alpha.

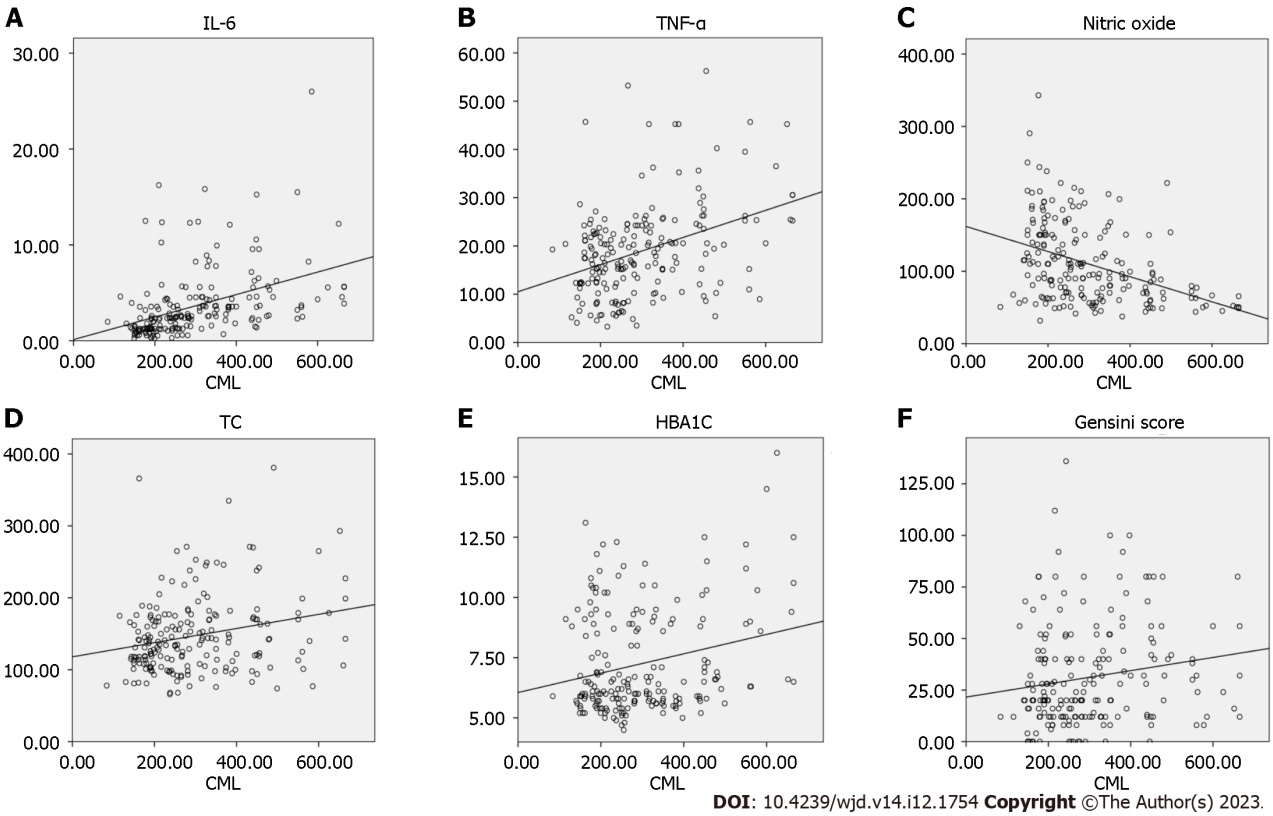

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Figure 2 Linear regression analysis of Nε-carboxymethyl-lysine with interleukin 6, tumor necrosis factor alpha, nitric oxide, total cholesterol, glycosylated hemoglobin, and Gensini Score. A: Regression line between Nε-carboxymethyl-lysine (CML) and interleukin-6 (IL-6); B: Regression line between CML and tumor necrosis factor-alpha (TNF-α); C: Regression line between CML and nitric oxide; D: Regression line between CML and total cholesterol (TC); E: Regression line between CML and glycosylated hemoglobin (HbA1c); F: Regression line between CML and Gensini score.

Similarly, Kerkeni *et al*[26] suggested that the serum concentrations of AGEs (CML and pentosidine) were significantly elevated in patients with CAD. Furthermore, serum pentosidine levels are independently associated with the occurrence of CAD with odds of 1.52. Additionally, the optimal cutoff value for pentosidine to predict the presence of CAD was found to be 3.2 μmol/mol[26].

Gaens *et al*[27] suggested that CML upregulates RAGE-dependent inflammatory responses and increases serum IL-6 level and TNF-α, which are negatively associated with serum nitric oxide and a high body mass index. Further in logistic regression analysis we found the CML level (264.43-364.31 ng/mL) significantly increased the risk of diabetic CAD. Similarly, Semba *et al*[28] suggested that in non-diabetic subjects serum CML was associated with anemia (odds ratio 1.33, 95%CI: 1.03-1.72, P = 0.029) in a multivariate logistic regression model, adjusting for age, sex, race, smoking, coronary heart disease, HF, and renal insufficiency. Kralev *et al*[29] suggested that a cutoff value of CML > 9.5 AU/mg was associated with an odds ratio of acute myocardial infarction of 39.7.

CONCLUSION

In conclusion, this study provided evidence for the association of CML and inflammatory markers with CAD in diabetic and non-diabetic patients. The results suggested that CML, IL-6, and TNF-α may be potential biomarkers for the prediction of CAD in diabetic patients, while nitric oxide may be a potential biomarker for the prediction of CAD in non-diabetic patients. These findings have significant clinical implications for the early diagnosis and management of CAD, particularly in diabetic patients who are at higher risk for developing cardiovascular complications. Further research on a larger cohort is needed to validate these findings and explore the underlying mechanisms of CML and inflammatory markers in the development of CAD, which may be helpful developing therapeutic interventions further.

ARTICLE HIGHLIGHTS

Research background

Coronary artery disease (CAD) is a widespread global health issue, responsible for a significant number of deaths. India bears a substantial burden, contributing to approximately one-fifth of CAD-related fatalities. The development of CAD has been closely linked to the accumulation of Nε-carboxymethyl-lysine (CML) in the heart muscle, a phenomenon associated with fibrosis. Understanding the role of CML in CAD development is crucial for combating this life-threatening condition.

Research motivation

This study is motivated by the need to shed light on the factors contributing to CAD, especially in the context of diabetes. CAD is a complex disease, and understanding its underlying mechanisms can help in early diagnosis and more effective management. Diabetes is a significant risk factor for CAD, and investigating the interplay between CML, inflammatory markers, and CAD in individuals with and without diabetes can provide valuable insights into its pathogenesis.

Research objectives

The primary objective of this research was to evaluate the impact of CML and inflammatory markers on the biochemical and cardiovascular characteristics of CAD patients, differentiating between diabetic and non-diabetes patients. The study aimed to identify potential links between CML, diabetes, and CAD and to assess if these factors could serve as predictive biomarkers.

Research methods

To achieve these objectives, this study enrolled 200 consecutive CAD patients undergoing coronary angiography. The patients were categorized into two groups based on their serum glycosylated hemoglobin (HbA1c) levels, with diabetic CAD patients (group I) having HbA1c levels of ≥ 6.5 and non-diabetic CAD patients (group II) with HbA1c levels < 6.5. Various parameters, including lipoprotein levels, plasma HbA1c levels, CML, interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and nitric oxide levels, were analyzed to assess the differences between the two groups.

Research results

The study revealed several significant findings. Group I, comprising 81 males and 19 females, had a mean age of 54.2 ± 10.2 years, with a mean diabetes duration of 4.9 ± 2.2 years. Group II, consisting of 89 males and 11 females, had a mean age of 53.2 ± 10.3 years. Group I exhibited more severe CAD, with a higher percentage of patients suffering from triple vessel disease and more severe stenosis in the left anterior descending coronary artery compared to group II. Group I patients also had a larger left atrium diameter. Significantly, group I patients displayed higher levels of CML, TNF-α, and IL-6 and lower levels of nitric oxide compared to group II patients. The study also demonstrated strong correlations between CML and inflammatory markers, with CML showing a significant positive correlation with IL-6 (r = 0.596, P = 0.001) and TNF-α (r = 0.337, P = 0.001) and a negative correlation with nitric oxide (r=-4.16, P = 0.001). Odds ratio analysis indicated that patients with CML in the third quartile (264.43-364.31 ng/mL) were significantly associated with diabetic CAD at both unadjusted and adjusted levels when considering various covariates.

Research conclusions

CML and inflammatory markers, particularly IL-6 and TNF-α, may play a significant role in the development of CAD, especially in individuals with diabetes. These findings suggest that CML and inflammatory markers can serve as potential biomarkers for predicting CAD, not only in diabetic patients but also in non-diabetic individuals. Understanding the mechanisms linking CML and inflammation to CAD provides valuable insights for improved CAD diagnosis, risk assessment, and management, which can ultimately contribute to reducing the burden of this life-threatening disease.

Research perspectives

Future studies should explore interventions targeting CML and inflammatory markers to mitigate CAD risk. Investigating therapeutic strategies and diagnostic tools based on these biomarkers can aid in early CAD detection and personalized treatment, potentially reducing CAD-related mortality rates globally.

FOOTNOTES

Co-corresponding authors: Pradeep Kumar Dabla and Desh Deepak Singh.

Author contributions: Dabla PK and Singh DD conceived, designed the study protocol; Shrivastav D, Dabla PK and Mehta V were involved in the data collection; Shrivastav D, Dabla PK, Singh DD, Mir R, Mehta V and Mehra P analyzed the data; Shrivastav D drafted the manuscript; Dabla PK, Singh DD, Mir R, Mehta V and Mehra P refined the manuscript; All authors were involved in the critical review of the results and have contributed to, read, and approved the final manuscript. The reasons for designating Dabla PK and Singh DD as co-corresponding authors are that they conceived and designed the study protocol, the collaborative effort, the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper and ensuring effective communication post submission. Further, the overall research team encompassed authors with a variety of expertise and skills from different fields with important contributions to complete the study and the resultant paper. This promotes the most comprehensive and in-depth examination of the research topic, ultimately enriching readers' understanding by offering various expert perspectives. Dabla PK, Singh DD as co-corresponding authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, contributions, and diversity.

Institutional review board statement: The study was approved by the ethics committee of the Institutional Ethical Committee of Maulana Azad Medical College and associated hospitals, Delhi, India (F1/IEC/MAMC/85/03/21/no.422; Dt-30.08.2021).

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at pradeep_dabla@yahoo.com.

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ORIGINAL ARTICLE

Case Control Study

Comparative study of type 2 diabetes mellitus-associated gut microbiota between the Dai and Han populations

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Abstract

BACKGROUND

The global prevalence of type 2 diabetes mellitus (T2DM) is increasing. T2DM is associated with alterations of the gut microbiota, which can be affected by age, illness, and genetics. Previous studies revealed that there are discriminating microbiota compositions between the Dai and the Han populations. However, the specific gut microbiota differences between the two populations have not been elucidated.

AIM

To compare the gut microbiota differences in subjects with and without T2DM in the Dai and Han populations.

METHODS

A total of 35 subjects of the Han population (including 15 healthy children, 8 adult healthy controls, and 12 adult T2DM patients) and 32 subjects of the Dai population (including 10 healthy children, 10 adult healthy controls, and 12 adult T2DM patients) were enrolled in this study. Fasting venous blood samples were collected from all the subjects for biochemical analysis. Fecal samples were collected from all the subjects for DNA extraction and 16S rRNA sequencing, which was followed by analyses of the gut microbiota composition.

RESULTS

No significant difference in alpha diversity was observed between healthy children and adults. The diversity of gut microbiota was decreased in T2DM patients compared to the healthy adults in both the Dai and Han populations.

There was a significant difference in gut microbiota between healthy children and healthy adults in the Han population with an increased abundance of Bacteroidetes and decreased Firmicutes in children. However, this difference was less in the Dai population. Significant increases in Bacteroidetes in the Han population and Proteobacteria in the Dai population and decreases in Firmicutes in both the Han and Dai population were observed in T2DM patients compared to healthy adults. Linear discriminant analysis Effect Size analysis also showed that the gut microbiota was different between the Han and Dai populations in heathy children, adults, and T2DM patients. Four bacteria were consistently increased and two consistently decreased in the Han population compared to the Dai population.

CONCLUSION

Differences in gut microbiota were found between the Han and Dai populations. A significant increase in Bacteroidetes was related to the occurrence of T2DM in the Han population, while a significant increase in Proteobacteria was related to the occurrence of T2DM in the Dai population.

Key Words: Gut microbiota; Type 2 diabetes mellitus; Dai population; Han population; Genetics; Ethnic

Core Tip: This study revealed that gut microbiota in the Han population is significantly different from the Dai population in healthy children, healthy adults, and patients with type 2 diabetes mellitus (T2DM). There was a significant difference in gut microbiota between healthy children and healthy adults in the Han population, but the difference was less in the Dai population. A significant increase in Bacteroidetes was observed in T2DM patients in the Han population, while a significant increase in Proteobacteria was observed in T2DM patients in the Dai population when compared to healthy controls.

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INTRODUCTION

As the world population and the proportion of elderly people increases, type 2 diabetes mellitus (T2DM), a prevalent metabolic disorder, has become a major global public health problem[1]. It is clinically characterized by hyperinsulinemia, insulin resistance, and islet cell damage, which can reach 50% at the time of diagnosis[2]. Individuals with T2DM are highly susceptible to vascular and neurological consequences in addition to life, psychological, and financial stress[3]. In 2021, 537 million people were diagnosed with diabetes, which is expected to increase to 643 million by 2030 and to 783 million by 2045[4]. Although the etiology and pathogenesis of T2DM are still unclear, recent studies have shown that gut microbiota may play key roles[5-8]. Identifying the features of gut microbiota associated with T2DM could help to better understand the pathogenesis of T2DM and prevent or delay the onset of the disease.

The gut microbiota is a complex microecological community composed of more than 100 trillion microorganisms[9-11]. However, it can be affected by various factors, such as age, diet, illness, environment, and genetics[12-14]. Different ethnic groups have a wide variety of dietary patterns, lifestyles, and geographical environments, which can lead to different presentations of gut microbiota and T2DM[15]. Previous studies revealed that there are discriminating microbiota compositions between the Dai and Han populations[16-18]. However, due to the heterogeneity among different ethnic groups, the results of these studies are difficult to replicate. Therefore, it is necessary to carry out analyses of gut microbiota in various ethnic groups with different genetic backgrounds in China.

The Dai ethnic group is a unique minority in Yunnan, China. They have lived in the valley area for generations and generally have endogamous marriages[19]. Due to the different genetic backgrounds, unique lifestyles, and geographical environments[19-22] of the Dai and Han populations, we hypothesized that there may be some underlying differences in the gut microbiota of the two populations. This study was designed to compare the gut microbiota in subjects with and without T2DM in the Dai and Han populations in Yunnan, China. The results of this investigation will help elucidate the underlying differences of gut microbiota between the Dai and Han populations and determine the association between gut microbiota and T2DM prevalence.

MATERIALS AND METHODS

Subjects recruitment

Healthy children, adult T2DM patients, and ethnically matched healthy adults from the Han and Dai populations living in the same area were recruited and enrolled in this study. Patients with T2DM met the following diagnostic criteria[23]: (1) Fasting blood glucose ≥ 7.0 mmol/L; or (2) Hemoglobin A1c ≥ 6.5%. The enrolled T2DM patients were newly diagnosed and drug-naïve. Subjects who had been treated with antibiotics in the previous 3 mo, were pregnant or lactating, or had inflammatory bowel disease were excluded from the study. The T2DM patients and healthy adults in each population were age-matched ($P > 0.05$). The enrolled subjects have completed the Study Questionnaires to provide personal details and information about health, diet, smoking activity and lifestyle at the time of sample collection. The study was approved by the ethics committee of the Sixth Affiliated Hospital of Kunming Medical University (approval no. 2023-kmykdx6f-66). All participants provided written informed consent.

Sample collection

Fasting venous blood samples were collected from all participants in the morning. After centrifugation (3000 rpm) at 4 °C for 10 min, the serum was immediately extracted and aliquoted. All blood and serum samples were stored at -80 °C until further biochemical analysis was performed. Stool samples were collected into 5-mL disposable sterile tubes within 30 min of discharge and kept at -80 °C until further processing was conducted.

Genomic DNA extraction and 16S rRNA gene sequencing

Total genomic DNA samples were extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, United States) according to the manufacturer's instructions. The quantity and quality of extracted DNA were measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and agarose gel electrophoresis, respectively. Prepared DNA samples were stored at -20 °C.

The V3-V4 region of 16S rRNA was amplified by polymerase chain reaction (PCR) with the primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The following thermal cycling conditions were utilized: initial denaturation at 98 °C for 5 min; 25 cycles consisting of denaturation at 98 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 45 s; and a final extension of 5 min at 72 °C. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, United States). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2 × 250 bp sequencing was performed using the Illlumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

Sequence and bioinformatics analyses

Microbiome bioinformatics were performed with QIIME2 2019.4 with slight modifications according to the official tutorials (https://docs.qiime2.org/2019.4/tutorials/). Briefly, raw sequence data were demultiplexed using the demux plugin followed by primer cutting with cutadapt plugin. Sequences were then quality filtered, denoised, merged, and chimera removed using the DADA2 plugin. Non-singleton amplicon sequence variants were aligned with mafft and used to construct a phylogeny with fasttree2. Taxonomy was assigned to amplicon sequence variants using the classify-sklearn naïve Bayes taxonomy classifier in feature-classifier plugin. Microbiota comparisons were performed using principal coordinate analysis (PCoA), principal component analysis (PCA), and permutational multivariate analysis of variance (PERMANOVA), diversity index estimate and composition of microbiome analysis.

Biochemical data analysis

Data were analyzed with SPSS Software (Version 29.0; IBM Corp., Armonk, NY, United States). Continuous data were expressed as mean ± SD. Comparisons between groups were performed using one-way analysis of variance and Student's *t* test. Cases with missing data for analysis were omitted, and the remaining data were analyzed. $P < 0.05$ was considered statistically significant.

RESULTS

Subject characteristics

A total of 35 subjects from the Han population (including 15 healthy children, 8 adult healthy controls, and 12 T2DM patients) and 32 subjects from the Dai population (including 10 healthy children, 10 adult healthy controls, and 12 T2DM patients) were enrolled in this study. The main demographics and blood biochemical indexes of the subjects are presented in Table 1. The levels of total bilirubin, direct bilirubin, indirect bilirubin, total cholesterol, hemoglobin A1c, gamma glutamyl transferase, and hypersensitive C-reactive protein were significantly higher in the T2DM patients in the Han population compared to the T2DM patients in the Dai population. Low density lipoprotein cholesterol and apolipoprotein A1 were significantly lower in T2DM patients in the Han population compared to T2DM patients in the Dai population. The levels of triglycerides, total bilirubin, indirect bilirubin, and gamma glutamyl transferase were significantly higher in healthy adults in the Dai population compared to healthy adults in the Han population.

Overall gut microbiota distribution in the Han and Dai populations

The total amount of data comprised 4505369 reads, with an average of 68736 reads per sample. For the 35 Han individuals, the total amount of data included 2132603 reads, with an average of 60931 reads per sample. For the 32 Dai individuals, the total amount of data included 2472766 reads, with an average of 77274 reads per sample. By clustering analysis at a 97% similarity, 22890 operational taxonomic units were identified.

Analysis of alpha diversity in gut microbiota

Six indices (Chao1, abundance-based coverage estimator, Good's coverage, Shannon, Simpson, and Pielou) were compared between all the population groups to evaluate the alpha diversity in gut microbiota. No significant difference in alpha diversity was observed between healthy children and adults. However, the diversity of the gut microbiota was decreased in T2DM patients compared to healthy adults in both the Dai and Han populations (Figure 1).

Comparison of gut microbiota between healthy children and healthy adults in the Han and Dai populations

PCA scores plot showed that there's differences among groups (Supplementary Figure 1). Further PCoA and PERMANOVA ($P < 0.05$) showed a clear distinction in the gut microbiota between healthy children and healthy adults in the Han population (Figure 2A). The gut microbiota composition of healthy children and healthy adults in the Han population was compared by the Linear discriminant analysis Effect Size (LEfSe) analysis (Figure 2B). The Bacteroidetes phylum was more abundant in the children of the Han population. In the Dai population, there was no clear distinction in the gut microbiota between healthy children and healthy adults after the PCoA (Figure 2C) and the LEfSe analysis (Figure 2D).

These data indicated that the difference in the gut microbiota between healthy children and healthy adults was greater in the Han population than in the Dai population and suggest that the influence of age, diet, or lifestyle on the gut microbiota is greater in the Han population than the Dai population.

Comparison of gut microbiota between healthy adults and adult T2DM patients in the Han and Dai populations

An obvious distinction between healthy adults and T2DM patients in the Han population was observed on the PCoA plot (Figure 3A). The gut microbiota composition of healthy adults and T2DM patients in the Han population was presented in a cladogram (Figure 3B). Compared with healthy adults, the T2DM patients from the Han population had an increased abundance of *Bacteroidetes*, *Bacteroidales*, *Megamonas*, and *Bacteroidia* within the Bacteroidetes phylum (Table 2).

There were no observable differences in the gut microbiota between healthy adults and T2DM patients in the Dai population after the PCoA (Figure 3C) and LEfSe analysis (Figure 3D). Compared with healthy adults, the T2DM patients from the Dai population had an increased abundance of *Deltaproteobacteria*, *Shigella*, and *Acinetobacter* within the Proteobacteria phylum (Table 3).

There were several bacterial types with a decreased abundance in T2DM patients in the Han population (63 total types) and in the Dai population (40 total types). Five of these bacteria, including *Dorea*, *Peptostreptococcaceae*, *Blautia*, *Ruminococcus*, and *Coprococcus*, were decreased in both populations (Figure 4).

Differences of gut microbiota between the Han and Dai populations during the transition from healthy children to healthy adults and T2DM

To investigate the differences of gut microbiota between the Han and Dai populations during the transition from healthy children to healthy adults and T2DM, the LEfSe analysis was performed (Figures 5-7). Compared to the Dai population, the abundances of *Coprococcus*, *Streptococcus*, and *Lactococcus* within the phylum Firmicutes were consistently higher in the Han population. We also observed that the abundances of *Rhizobiales* within the phylum Proteobacteria and *Veillonellaceae* within the phylum Firmicutes were significantly lower in the Han population (Table 4).

DISCUSSION

The relationship between gut microbiota and T2DM is becoming increasingly important. In the past decade, studies have supported the role of gut microbiota in the pathogenesis of T2DM[24-28]. Some researchers have reported that there are discriminating microbiota compositions between the Han and the Tibetans populations[16-18] and also different among the different ethnicities: Han, Zang, Bai, Hani, Dai, and Miao (including both healthy urban and rural residents of each ethnicity)[29]. However, the underlying differences of the gut microbiota between the Han and Dai populations have not been elucidated. Here, we performed a comparative analysis of the gut microbiota in subjects with and without T2DM from the Dai and Han populations in Yunnan Province, China. To the best of our knowledge, this is the first time to compare the T2DM-associated gut microbiota between Han and Dai populations.

The alpha diversity of the gut microbiota, which reflects the abundance, evenness, and richness[30], might vary between ethnic groups in part due to the varied prevalence of T2DM among ethnic groups[31]. Interestingly, our study showed that there was no significant difference in alpha diversity between the Han and Dai populations, suggesting that the abundance, evenness, and richness of the gut microbiota were not significantly different between the Han and Dai populations. However, the diversity of gut microbiota was decreased in T2DM patients compared to healthy adults in both the Han and Dai populations (Figure 1), which is consistent with the previous results in different populations of the world, including other populations in China[32-34].

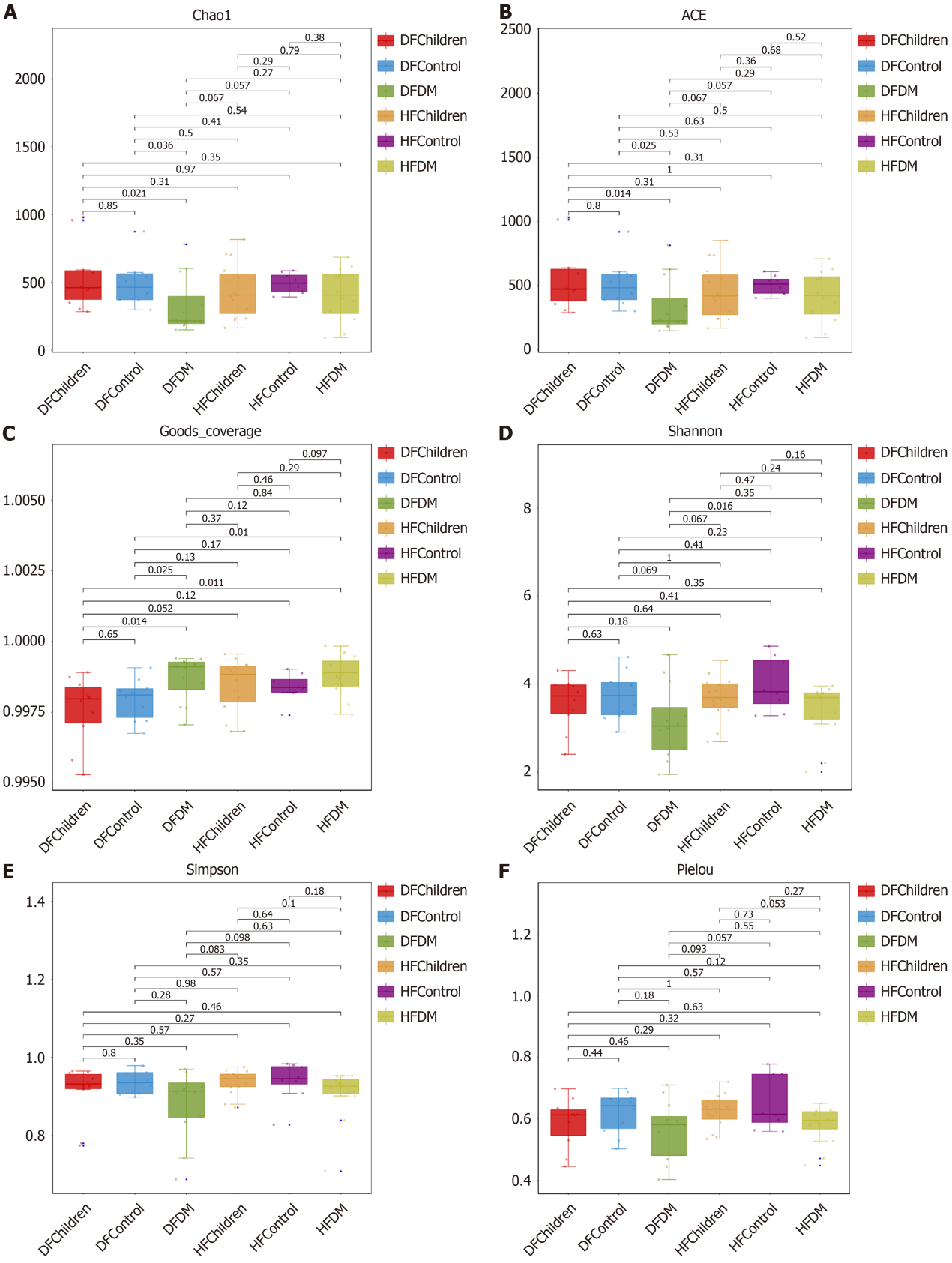

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Figure 1 Analysis of alpha diversity in the gut microbiota. We evaluated and compared six indices of alpha diversity. A: Chao1 index; B: Abundance-based coverage estimator index; C: Good's coverage index; D: Shannon index; E: Simpson index; F: Pielou index. There were no significant differences observed in the population groups among the six indices ($P > 0.05$). ACE: Abundance-based coverage estimator; DFChildren: Healthy children from the Dai population; DFControl: Healthy adults from the Dai population; DFDM: Adult type 2 diabetes mellitus patients from the Dai population; HFChildren: Healthy children from the Han population; HFControl: Healthy adults from the Han population; HFDM: Adult type 2 diabetes mellitus patients from the Han population.

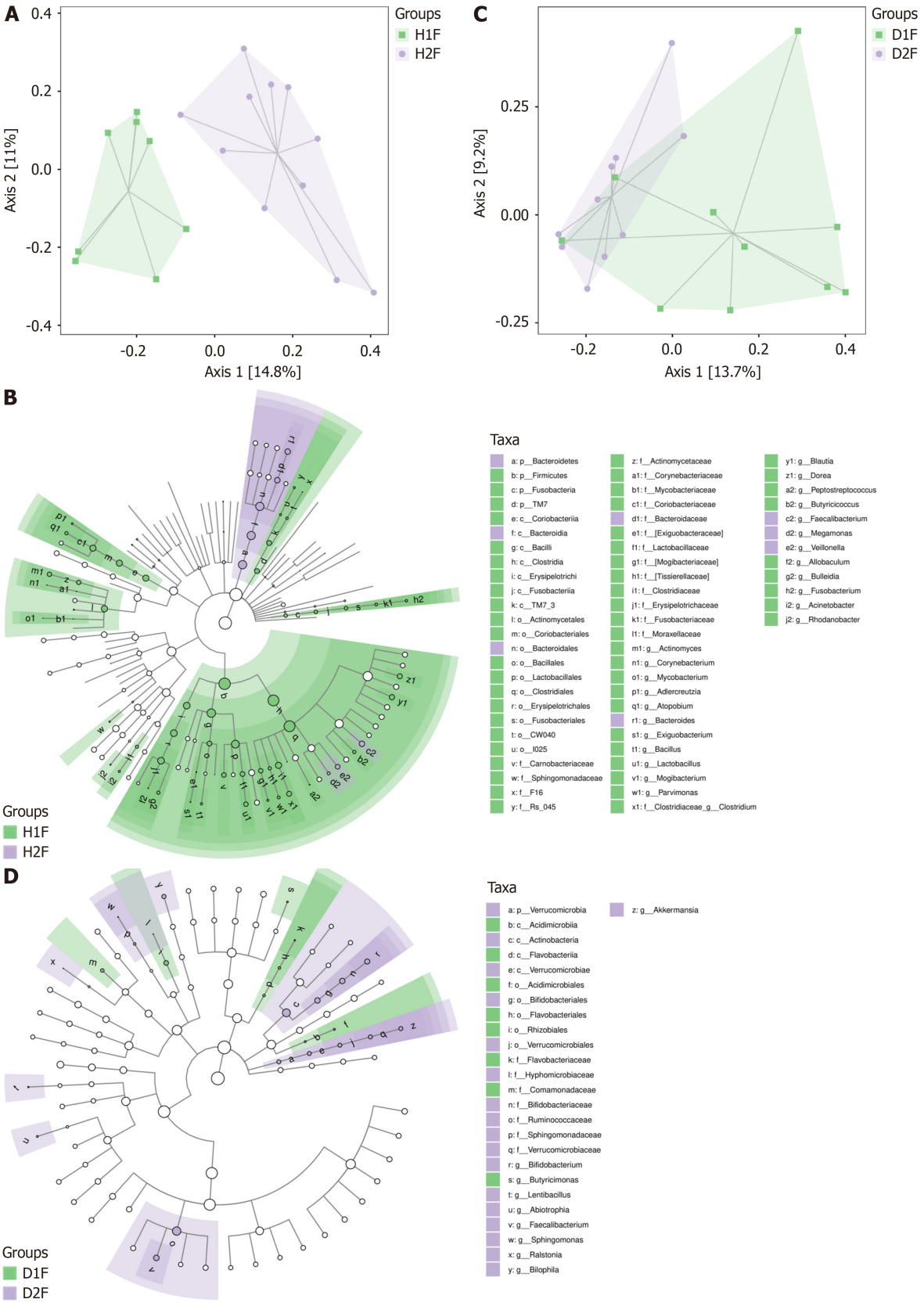

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Figure 2 Comparison of gut microbiota between healthy children and healthy adults in the Han and Dai populations. A: Clustering of gut microbiota composition between the healthy children and healthy adults in the Han population. There was a significant difference and clear distinction between the groups ($P < 0.05$); B: Gut microbiota composition analysis between healthy children and healthy adults in the Han population; C: Clustering of gut microbiota composition between healthy children and adults in the Dai population. There was no significant difference between the groups ($P > 0.05$); D: Gut microbiota composition analysis between healthy children and healthy adults in the Dai population. H1F: Healthy adults in the Han population; H2F: Healthy children in the Han population; D1F: Healthy adults in the Dai population; D2F: Healthy children in the Dai population.

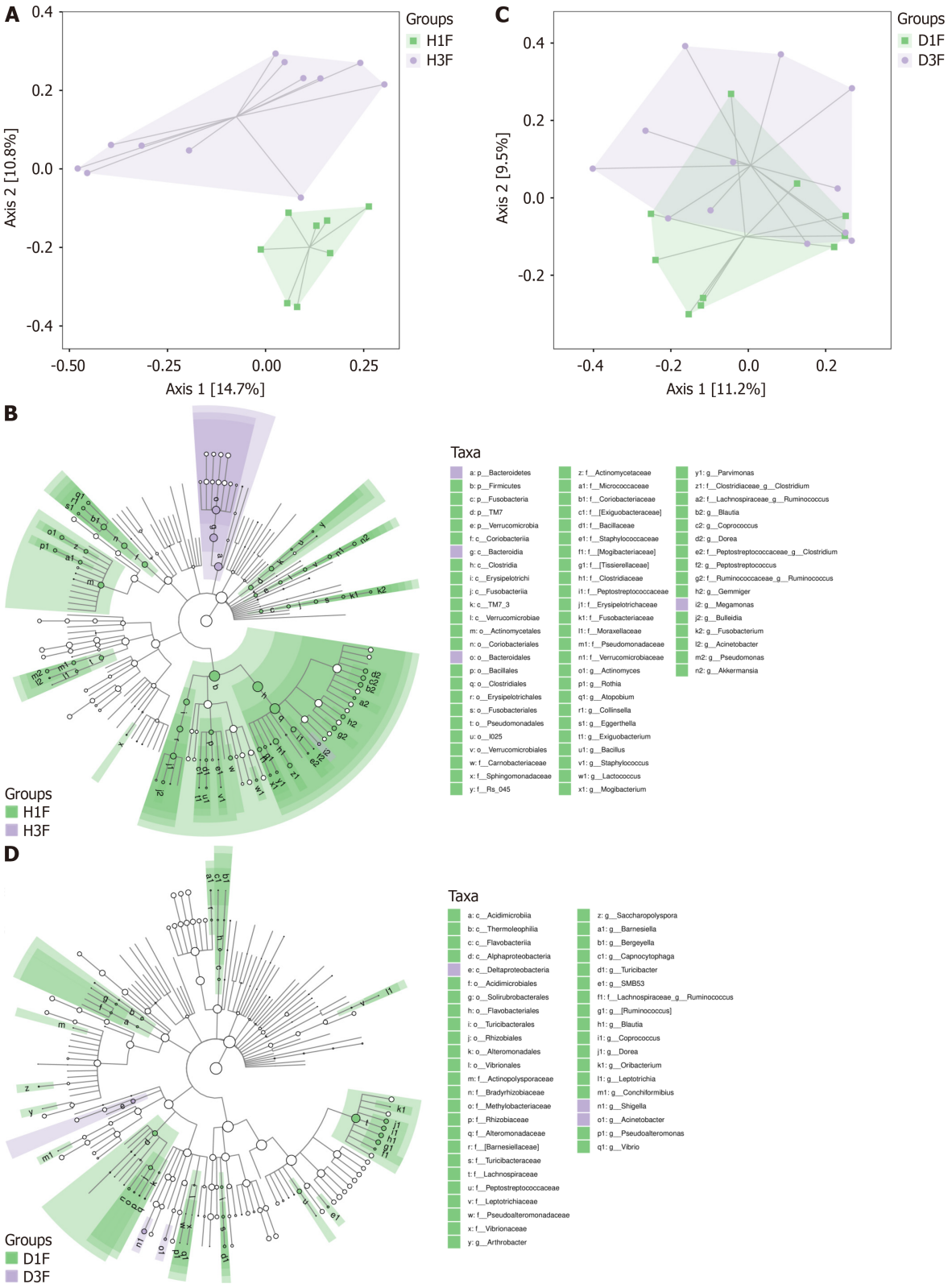

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Figure 3 Comparison of gut microbiota between healthy adults and type 2 diabetes mellitus patients in the Han and Dai populations. A: Clustering of gut microbiota composition between the healthy adults and type 2 diabetes mellitus (T2DM) patients in the Han population. There was a significant difference between the two groups ($P < 0.05$); B: Gut microbiota composition analysis between the healthy adults and T2DM patients in the Han population. There was an increased abundance of *Bacteroidetes, Bacteroidales, Megamonas* and *Bacteroidia* in the T2DM patients ($P < 0.05$); C: Clustering of gut microbiota composition between the healthy adults and T2DM patients in the Dai population. There were no significant differences between two groups ($P > 0.05$); D: Gut microbiota composition analysis between the healthy adults and T2DM patients in the Dai population. There was an increased abundance of *Deltaproteobacteria, Shigella*, and *Acinetobacter* ($P < 0.05$). D1F: Healthy adults in the Dai population; D3F: Type 2 diabetes mellitus patients in the Dai population; H1F: Healthy adults in the Han population; H3F: Type 2 diabetes mellitus patients in the Han population.

Table 1 Subject demographics and biochemical blood indices

Parameter	Han population			Dai population		
	Healthy adults, *n* = 8	T2DM patients, *n* = 12	Healthy children, *n* = 15	Healthy adults, *n* = 10	T2DM patients, *n* = 12	Healthy children, *n* = 10
Sex as male/female	3/5	8/4	7/8	5/5	9/3	4/6
Age in yr	50.63 ± 5.85	55.27 ± 11.76	5.00 ± 2.10[1]	45.70 ± 14.66[2]	55.92 ± 9.07	5.50 ± 1.18[3]
FBG in mmol/L	4.85 ± 0.38	9.98 ± 5.89[1]	3.23 ± 0.99	5.62 ± 1.36	7.74 ± 2.39[3]	4.52 ± 0.29
HbA1c as %	5.55 ± 0.24	8.55 ± 2.59[1]	5.23 ± 0.15	5.58 ± 0.19	7.24 ± 0.77[3,4]	5.19 ± 0.35
TG in mmol/L	1.90 ± 0.63	2.22 ± 1.54	0.97 ± 0.24[1]	3.90 ± 4.10[2]	2.48 ± 1.91	1.12 ± 0.45[3]
TC in mmol/L	5.15 ± 0.61	3.76 ± 1.45[1]	4.01 ± 0.88	5.51 ± 1.04	4.85 ± 1.26[4]	3.91 ± 0.57[3]
HDL-C in mmol/L	1.28 ± 0.28	0.97 ± 0.22[1]	1.48 ± 0.34	1.23 ± 0.28	1.26 ± 0.40	1.39 ± 0.42
LDL-C in mmol/L	3.12 ± 0.64	1.82 ± 0.71[1]	2.16 ± 0.63[1]	2.98 ± 1.23	2.75 ± 1.05[4]	2.16 ± 0.38
APO-A1 in g/L	1.75 ± 0.27	1.30 ± 0.30[1]	1.75 ± 0.36	1.83 ± 0.23	1.74 ± 0.37[4]	1.61 ± 0.36
APO-B in g/L	1.04 ± 0.15	0.68 ± 0.18[1]	0.72 ± 0.19[1]	0.98 ± 0.24	0.94 ± 0.29	0.66 ± 0.09[3]
WBC as 10^9/L	6.06 ± 1.44	6.41 ± 1.87	6.95 ± 1.11	6.79 ± 1.43	7.07 ± 2.09	8.32 ± 2.38
RBC as 10^{12}/L	5.19 ± 0.66	4.82 ± 0.57	4.89 ± 0.27	5.09 ± 0.65	5.05 ± 0.57	4.90 ± 0.42
TBil in μmol/L	6.84 ± 1.59	14.19 ± 9.35[1]	6.07 ± 4.48	8.71 ± 5.08[2]	6.19 ± 1.98[4]	5.74 ± 2.38
DBil in μmol/L	3.84 ± 0.79	6.31 ± 2.92[1]	2.92 ± 2.34	3.96 ± 2.05	3.35 ± 1.02[4]	3.13 ± 1.40
IBil in μmol/L	3.00 ± 0.85	7.88 ± 6.51[1]	3.15 ± 2.28	4.75 ± 3.05[2]	2.84 ± 1.34[4]	2.61 ± 1.41[3]
ALT in U/L	22.50 ± 11.33	31.36 ± 26.34	15.80 ± 14.85	25.50 ± 15.68	26.10 ± 17.22	13.70 ± 14.96
AST in U/L	21.13 ± 4.58	27.36 ± 25.61	31.50 ± 7.18[1]	23.26 ± 8.71	23.14 ± 8.64	27.40 ± 6.45
GGT in U/L	34.75 ± 24.40	51.82 ± 90.65	9.67 ± 2.94[1]	70.40 ± 66.34[2]	38.67 ± 22.80[4]	13.50 ± 8.15[3]
BUN in mmol/L	5.63 ± 1.40	4.59 ± 1.70	3.68 ± 0.82[1]	5.12 ± 1.51	5.23 ± 2.18	3.75 ± 0.91
Cr in μmol/L	70.38 ± 8.75	69.55 ± 20.92	32.17 ± 7.60[1]	68.20 ± 17.69	85.50 ± 30.21	32.00 ± 4.00[3]
Hcy in μmol/L	15.63 ± 3.45	12.35 ± 1.55[1]	14.20 ± 3.12	13.59 ± 3.57	16.69 ± 3.47[4]	11.55 ± 1.53[5]
hs-CRP in mg/L	1.85 ± 3.35	3.46 ± 5.23	2.84 ± 6.66[5]	1.84 ± 1.57	1.83 ± 1.47[4]	0.47 ± 0.50[3]

[1]$P < 0.05$ *vs* healthy adults in the Han population.
[2]$P < 0.05$, healthy adults in the Han population *vs* healthy adults in the Dai population.
[3]$P < 0.05$ *vs* healthy adults in the Dai population.
[4]$P < 0.05$, adult type 2 diabetes mellitus patients in the Han population *vs* adult type 2 diabetes mellitus patients in the Dai population.
[5]$P < 0.05$, healthy children in the Han population *vs* healthy children in the Dai population.
ALT: Alanine transaminase; APO: Apolipoprotein; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; Cr: Creatinine; DBil: Direct bilirubin; FBG: Fasting blood glucose; GGT: Gamma glutamyl transferase; HbA1c: Hemoglobin A1c; Hcy: Homocysteine; HDL-C: High density lipoprotein cholesterol; hs-CRP: Hypersensitive C-reactive protein; IBil: Indirect bilirubin; LDL-C: Low density lipoprotein cholesterol; RBC: Red blood cell; T2DM: Type 2 diabetes mellitus; TBil: Total bilirubin; TC: Total cholesterol; TG: Triglyceride; WBC: White blood cell.

Table 2 Significantly increased bacteria in type 2 diabetes mellitus patients in the Han population

Taxa	Abundance, T2DM patients *vs* healthy adults	LDA score	*P* value
Bacteria. Bacteroidetes	5.353	4.939	0.037
Bacteria. Bacteroidetes. *Bacteroidia*. *Bacteroidales*	5.353	4.939	0.037
Bacteria. Firmicutes. *Clostridia*. *Clostridiales*. *Veillonellaceae*. *Megamonas*	4.347	3.760	0.045
Bacteria. Bacteroidetes. *Bacteroidia*	5.353	4.939	0.037

LDA: Linear discriminant analysis; T2DM: Type 2 diabetes mellitus.

Table 3 Significantly increased gut microbiota in type 2 diabetes mellitus patients in the Dai population

Taxa	Abundance, T2DM patients *vs* healthy adults	LDA score	*P* value
Bacteria. Proteobacteria. *Gammaproteobacteria*. *Pseudomonadales*. *Moraxellaceae*. *Acinetobacter*	2.047	2.754	0.004
Bacteria. Proteobacteria. *Gammaproteobacteria*. *Enterobacteriales*. *Enterobacteriaceae*. *Shigella*	4.711	4.288	0.021
Bacteria. Proteobacteria. *Deltaproteobacteria*	3.199	2.764	0.044

LDA: Linear discriminant analysis; T2DM: Type 2 diabetes mellitus.

Table 4 Differential gut microbiota observed in the Han population *vs* Dai population

Differential gut microbiota	Healthy children	Healthy adults	T2DM patients
Han population			
d__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Coprococcus.s__unidentified_Coprococcus	0.542[a]	0.259[a]	0.461
d__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Streptococcaceae.g__Streptococcus.s__unclassified_Streptococcus	0.000[a]	0.800[a]	1.000
d__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Coprococcus	0.521[a]	0.286[a]	0.475
d__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Streptococcaceae.g__Lactococcus	0.009[a]	0.019[a]	0.083
Dai population			
d__Bacteria.p__Proteobacteria.c__Alphaproteobacteria.o__Rhizobiales	4.840[a]	1.159[a]	1.636
d__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridiales.f__Veillonellaceae.g__unclassified_Veillonellaceae	8.800[a]	12.000[a]	1.688

[a]$P < 0.05$ Han population *vs* Dai population.
T2DM: Type 2 diabetes mellitus.

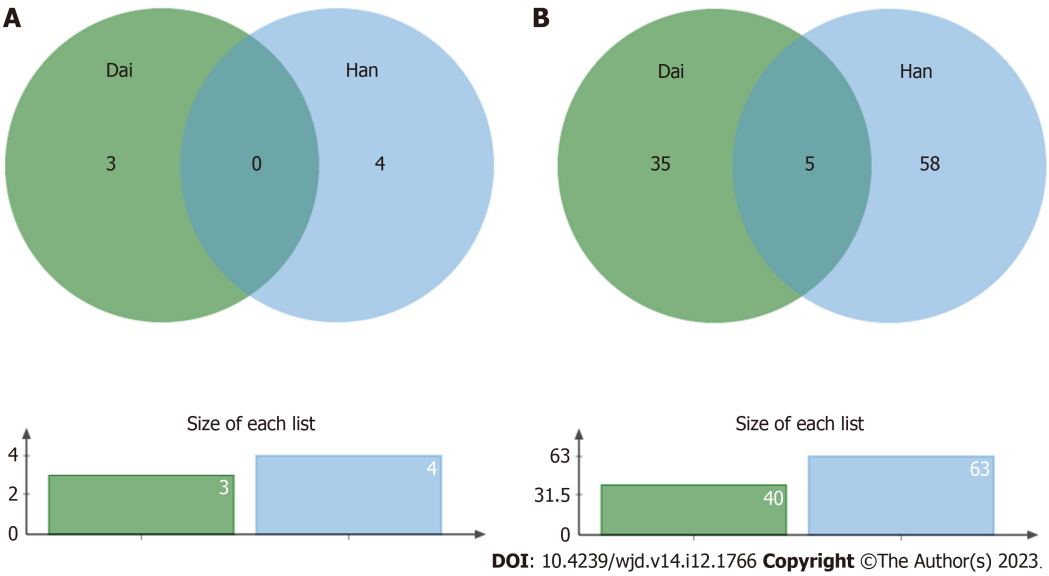

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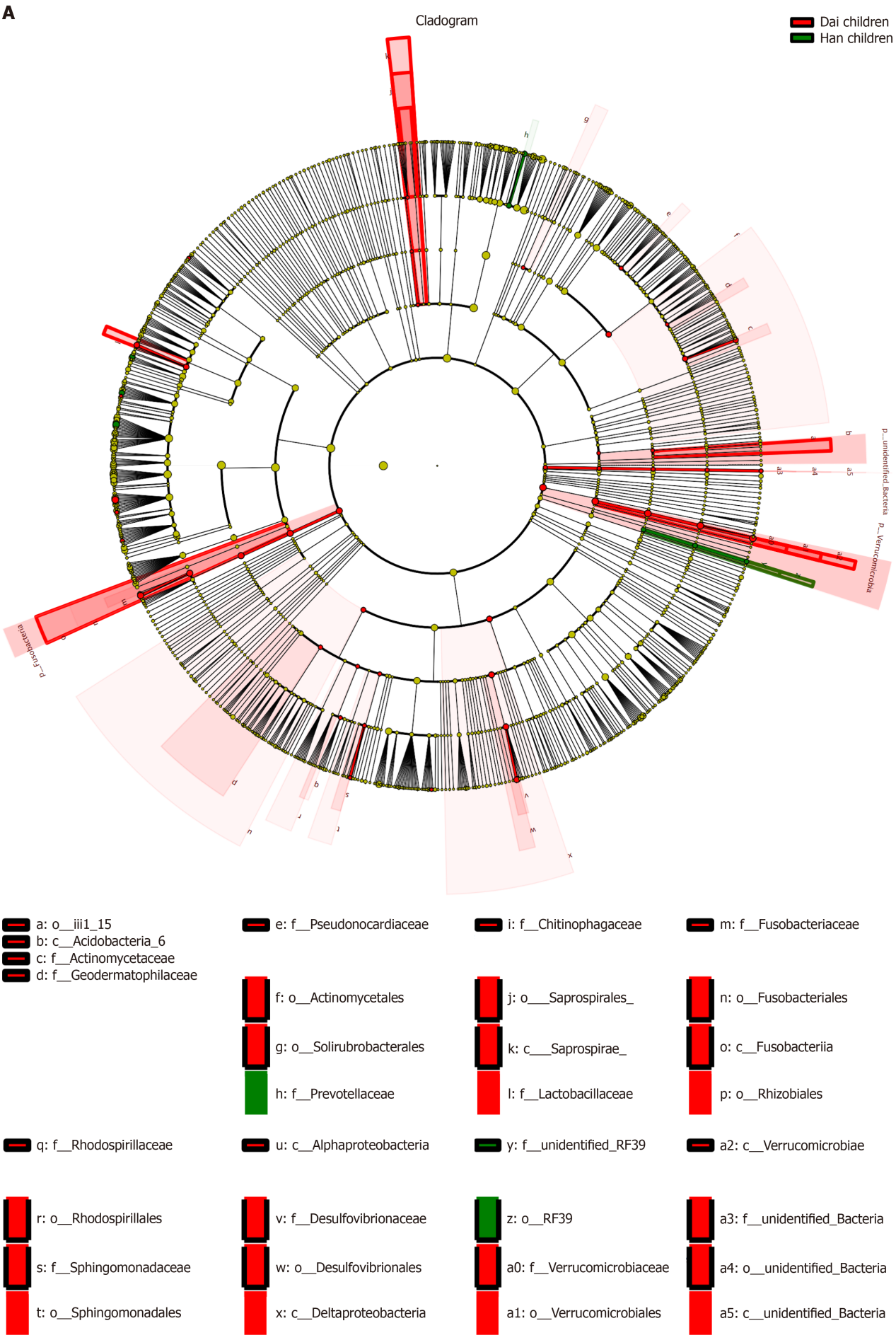
Figure 4 Venn diagram of the unique and shared bacteria in type 2 diabetes mellitus patients in the Han and Dai populations. A: Bacteria with increased abundance; B: Bacteria with decreased abundance. The five bacteria that were decreased in both the Han and Dai populations were *Dorea*, *Peptostreptococcaceae*, *Blautia*, *Ruminococcus*, and *Coprococcus*.


A
Cladogram
Dai children
Han children
p__unidentified_Bacteria
p__Verrucomicrobia
p__Fusobacteria
a: o__iii1_15
b: c__Acidobacteria_6
c: f__Actinomycetaceae
d: f__Geodermatophilaceae
e: f__Pseudonocardiaceae
f: o__Actinomycetales
g: o__Solirubrobacterales
h: f__Prevotellaceae
i: f__Chitinophagaceae
j: o___Saprospirales_
k: c___Saprospirae_
l: f__Lactobacillaceae
m: f__Fusobacteriaceae
n: o__Fusobacteriales
o: c__Fusobacteriia
p: o__Rhizobiales
q: f__Rhodospirillaceae
r: o__Rhodospirillales
s: f__Sphingomonadaceae
t: o__Sphingomonadales
u: c__Alphaproteobacteria
v: f__Desulfovibrionaceae
w: o__Desulfovibrionales
x: c__Deltaproteobacteria
y: f__unidentified_RF39
z: o__RF39
a0: f__Verrucomicrobiaceae
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a5: c__unidentified_Bacteria

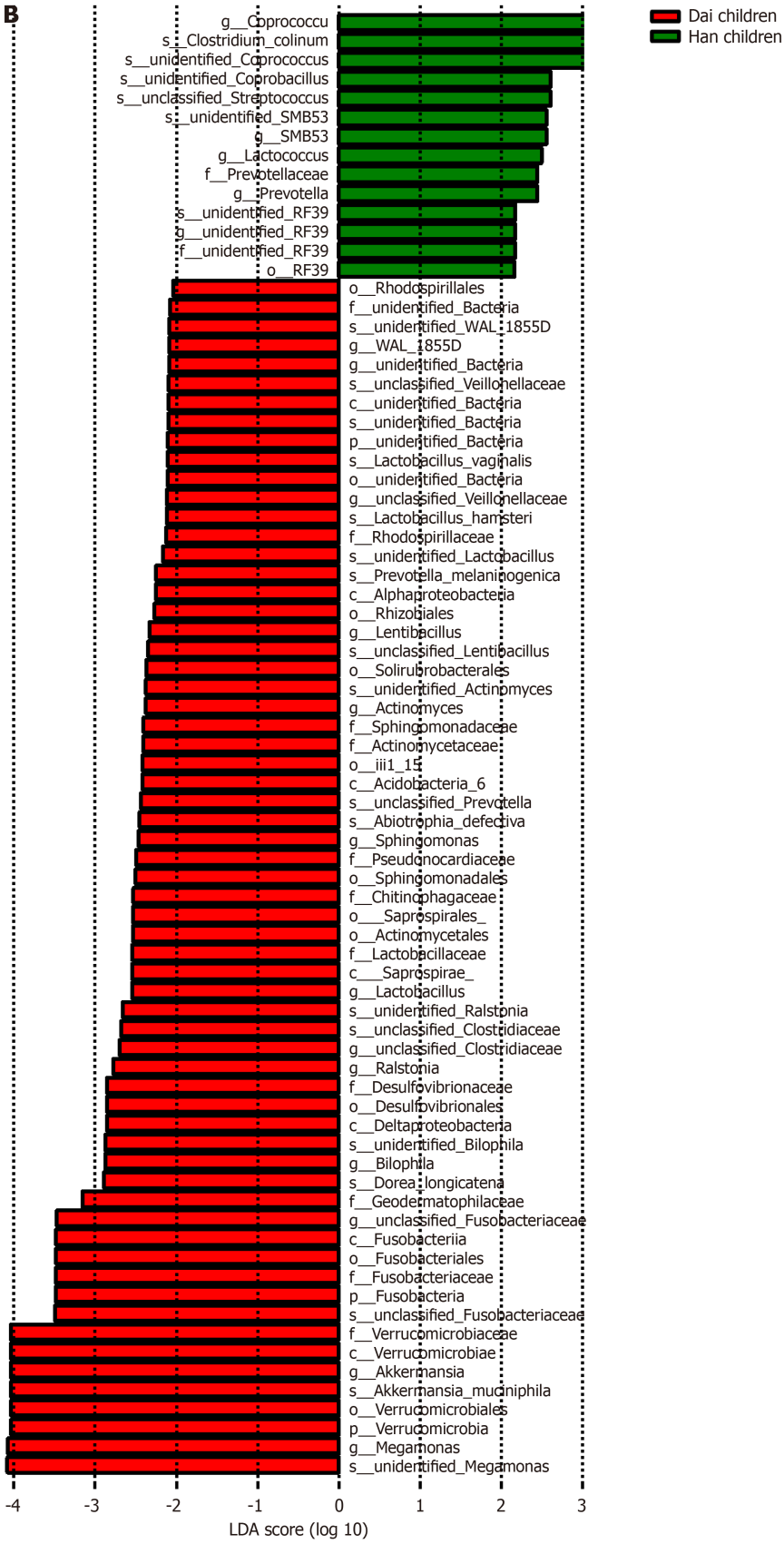

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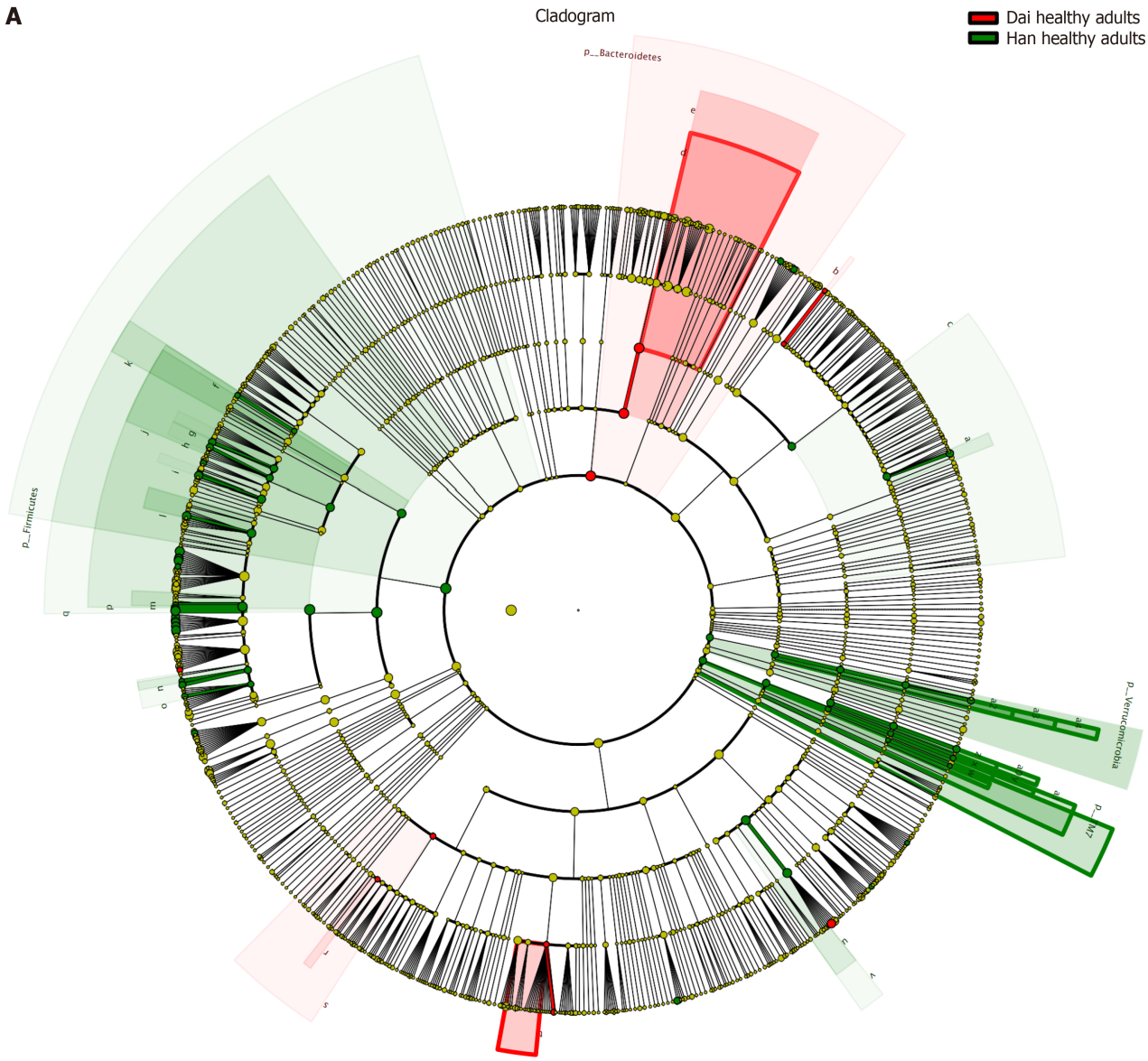
Figure 5 Cladogram of the Linear discriminant analysis Effect Size analysis in healthy children. A: Taxonomic representation of statistically and biologically consistent differences between the healthy children in the Han and Dai populations. Differences are represented in the color of the most abundant class (red indicates the Dai population, and green indicates the Han population). The diameter of each circle is proportional to the abundance of the taxon; B The Linear discriminant analysis Effect Size analysis provided a list of features that were statistically and biologically different (ranked by the effect size). T2DM: Type 2 diabetes mellitus.


A
Cladogram
Dai healthy adults
Han healthy adults
p__Bacteroidetes
p__Firmicutes
p__Verrucomicrobia
a: f__Actinomycetaceae
b: f__unidentified_Actinomycetales
c: o__Actinomycetales
d: o__Bacteroidales
e: c__Bacteroidia
i: f__Streptococcaceae
m: f__Peptostreptococcaceae
f: f___Exiguobacteraceae_
g: f__Carnobacteriaceae
h: f__Enterococcaceae
j: o__Lactobacillales
k: c__Bacilli
l: f__Clostridiaceae
n: f___Mogibacteriaceae_
o: f___Tissierellaceae_
p: o__Clostridiales
q: c__Clostridia
u: f__Enterobacteriaceae
y: o__I025
a2: f__Verrucomicrobiaceae
r: f__Bradyrhizobiaceae
s: o__Rhizobiales
t: f__Comamonadaceae
v: o__Enterobacteriales
w: f__F16
x: f__Rs_045
z: f__unidentified_TM7_3
a0: o__unidentified_
a1: c__TM7_3
a3: o__Verrucomicrobiales
a4: c__Verrucomicrobiae

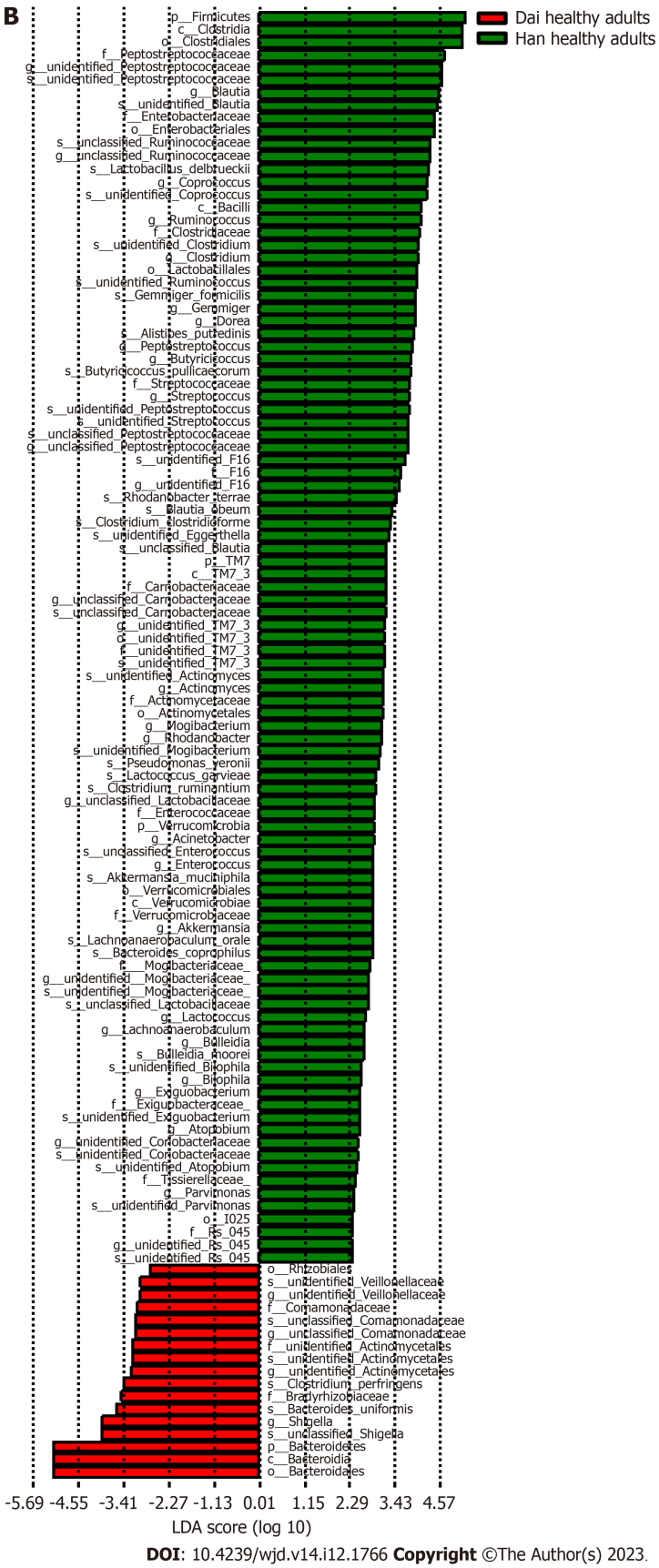


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Figure 6 Cladogram of the Linear discriminant analysis Effect Size analysis in healthy adults. A: Taxonomic representation of statistically and biologically consistent differences between the healthy adults in the Han and Dai populations. Differences are represented in the color of the most abundant class (red indicates the Dai population, and green indicates the Han population). The diameter of each circle is proportional to the abundance of the taxon; B The Linear discriminant analysis Effect Size analysis provided a list of features that were statistically and biologically different (ranked by the effect size). T2DM: Type 2 diabetes mellitus.

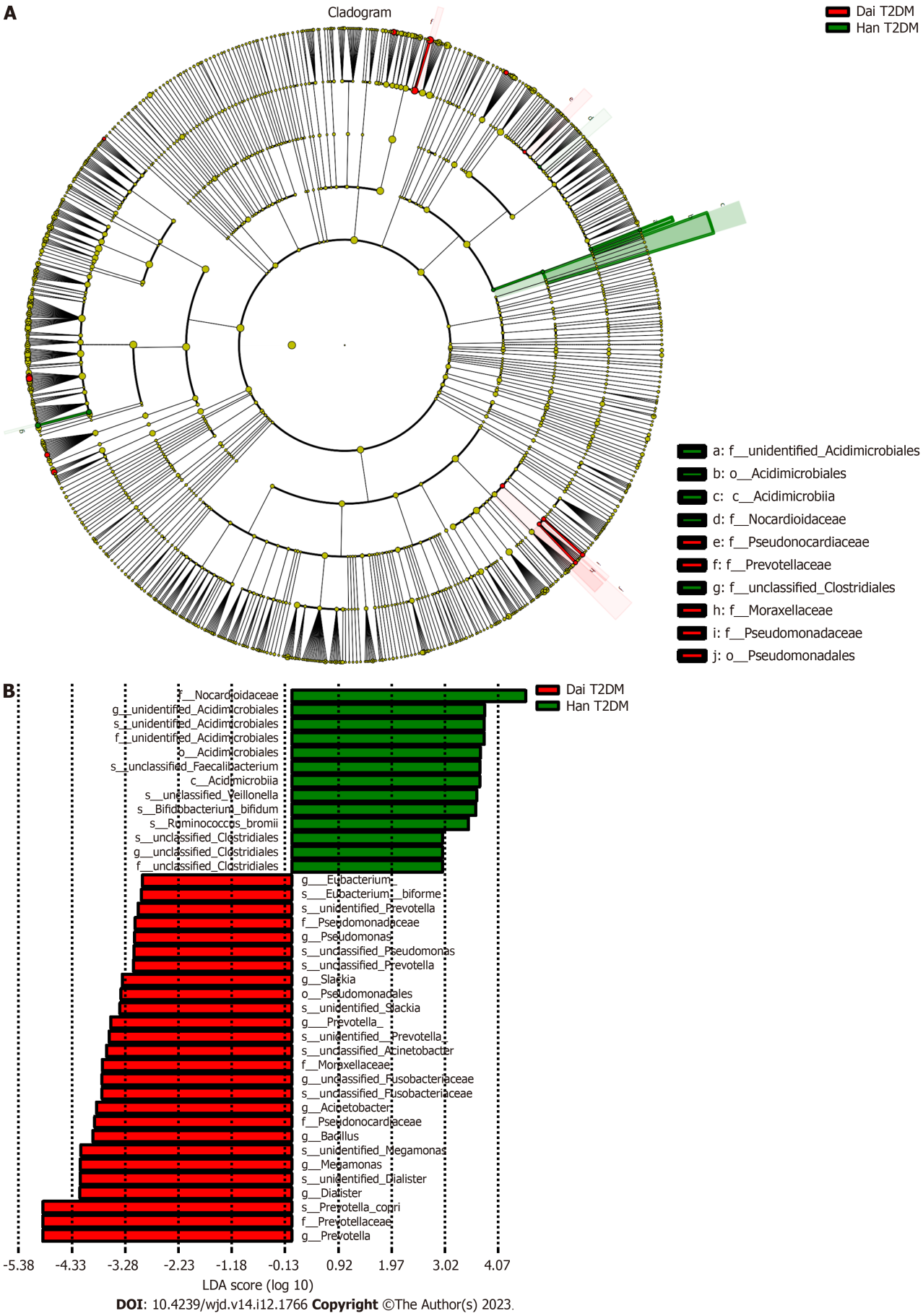

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Figure 7 Cladogram of the Linear discriminant analysis Effect Size analysis in type 2 diabetes mellitus patients. A: Taxonomic representation of statistically and biologically consistent differences between the type 2 diabetes mellitus patients in the Han and Dai populations. Differences are represented in the

color of the most abundant class (red indicates the Dai population, and green indicates the Han population). The diameter of each circle is proportional to the abundance of the taxon; B The Linear discriminant analysis Effect Size analysis provided a list of features that were statistically and biologically different (ranked by the effect size). T2DM: Type 2 diabetes mellitus.

The gut microbiota is associated with the age of host[35]. Despite being matched for age (significance less than 0.05), there was a large deviation from the mean value of the age of healthy adults with diabetes in Dai who were actually enrolled in the analysis. We also selected some of these samples with matched age means for subset analysis, and the results were consistent with the existing result. To determine the influence of age on gut microbiota, we also conducted a comparison of gut microbiota between healthy children and healthy adults in the Han and Dai populations. These findings suggested that the difference in the gut microbiota between healthy children and healthy adults was greater in the Han population than the Dai population. The observed higher relative abundances of genus *Bacteroides* in children and higher relative abundances of genus *Blautia* in adults were consistent with the previous studies[36].

Many researchers have reported that the gut microbiota diversity is affected by T2DM[26,37]. After comparing the gut microbiota between healthy adults and T2DM patients in the Han population, we observed a significant difference in the gut microbiota. However, there was no clear distinction between healthy adults and T2DM patients in the Dai population. The underlying reason behind this warrant further investigation. Moreover, our data showed that the T2DM patients of the Dai population possessed a distinctive microbiota composition characterized by a high abundance of Proteobacteria, which is consistent with the previous results[38]. Recent evidence has also shown that Proteobacteria in gut microbial dysbiosis is essential for metabolic disorders[39]. Interestingly, we found that T2DM patients from the Han population had an increase in *Bacteroidetes*, *Bacteroidales*, *Megamonas* and *Bacteroidia* within the phylum Bacteroidetes. This discovery conflicted with the results of some earlier studies[32,40]. The possible reason might be that the proportion of *Bacteroidales* abundance can be altered by high-calorie diets[41], which is also a possible cause of T2DM.

Although we have identified that both age and T2DM influence the gut microbiota, it is unknown which has a greater effect. We explored the differences in bacteria between healthy children to healthy adults and healthy adults to T2DM in both ethnic groups. The results showed that the differences of healthy children between the Han and Dai population were still significant in healthy adults. However, these changes in T2DM patients were not statistically significant. These results demonstrated that the differences were influenced more by age than T2DM during the transition from healthy children to healthy adults and T2DM patients in both the Han and Dai populations.

Several limitations of this study should be taken into account. First, the sample size was relatively small, which limits the generalizability of the findings. It should be confirmed in a larger scale of samples in the future. Second, the effect of gender, food, and smoking activity were not investigated in the study. Third, the metabolic profile requires further investigation to confirm the relationship between the imbalance of metabolism and gut microbiota alterations.

CONCLUSION

Through the comparative analysis, this study found significant differences in the gut microbiota in the Han and Dai populations, and these differences were influenced to a greater degree by age than by T2DM. Our findings may provide additional insight for further study of gut microbiota dysbiosis-related diseases in the Han and Dai populations.

ARTICLE HIGHLIGHTS

Research background

Previous studies revealed that there are discriminating microbiota compositions between the Han and Dai populations. However, the underlying differences in the gut microbiota between the Han and Dai populations have not yet been elucidated.

Research motivation

We compared the differences in the gut microbiota in subjects with and without type 2 diabetes mellitus (T2DM) in the Han and Dai populations to explore the pathogenic mechanism of T2DM.

Research objectives

To identify the differences in the gut microbiota related to the occurrence of T2DM in the Han and Dai populations.

Research methods

A total of 35 subjects of the Han population (15 healthy children, 8 adult healthy controls, and 12 adult T2DM patients) and 32 subjects of the Dai population (10 healthy children, 10 adult healthy controls, and 12 adult T2DM patients) were enrolled in this study. Fasting venous blood samples were collected from all the subjects for biochemical analysis. Fecal samples were collected from all the subjects for DNA extraction and 16S rRNA sequencing, which was followed by analyses of the gut microbiota composition.

Research results

Fasting plasma glucose levels and hemoglobin A1c were significantly increased in the T2DM patients. The gut microbiota of the Han population was significantly different from the Dai population in healthy children, healthy adults, and T2DM patients. Significant increases in Bacteroidetes were observed in T2DM patients from the Han population, while significant increases in Proteobacteria were observed in T2DM patients in the Dai population.

Research conclusions

We observed significant differences in the gut microbiota in the Han and Dai populations, and these differences were influenced to a greater degree by age than by T2DM.

Research perspectives

Our findings may provide additional insight for further study of the gut microbiota dysbiosis-related diseases in the Han and Dai populations. Future research should include a larger scale of samples and an investigation of the metabolic profile in order to confirm the relationship between an imbalance of the metabolism and gut microbiota alterations.

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FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study

Early hemodynamics after tibial transverse transport in patients with nonarterial stenosis and arterial stenosis diabetic foot

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Abstract

BACKGROUND

The diagnosis of peripheral arteriopathy in the diabetic foot is complicated by diabetes and its advanced complications. It has been found that diabetic foot can be categorized into arterial stenosis and non-arterial stenosis, both of which have significant differences in hemodynamic characteristics.

AIM

To evaluate the early hemodynamic changes in diabetic foot patients with nonarterial stenosis and arterial stenosis treated by tibial transverse transport (TTT) using high-frequency color Doppler ultrasonography (HFCDU) and a laser Doppler flowmeter.

METHODS

Twenty-five patients with Wagner grades 3-5 diabetic foot ulcers were treated with TTT, and the wound healing time and rate were recorded. Patients were grouped according to the results of preoperative lower-extremity ultrasonography. Cases with ≥ 50% stenosis in any of the femoral, popliteal, posterior tibial, anterior tibial, and peroneal arteries of the affected limb were classified as the arterial stenosis group (n = 16); otherwise, they were classified as the nonarterial stenosis group (n = 9). Before and one month after surgery, HFCDU was used to evaluate the degree of lower limb artery lesions and hemodynamic changes in patients. The degree of femoral-popliteal atherosclerotic stenosis, the degree of vascular stenosis and occlusion of the lower-knee outflow tract, and the degree of medial arterial calcification were scored; the three scores were added together to obtain the total score of lower extremity arteriopathy. PeriScanPIM3, a laser Doppler flowmeter system, was used to detect alterations in plantar microcirculation before and 1 mo after surgery. Wound healing and hemodynamic indices

were compared between the two groups.

RESULTS

The wound healing time of the diabetic foot was significantly shorter in the nonarterial stenosis group than in the arterial stenosis group (47.8 ± 13 *vs* 85.8 ± 26, $P < 0.05$), and the wound healing rate of both groups was 100%. The preoperative total lower extremity arteriopathy scores were lower in the nonarterial stenosis group than those in the arterial stenosis group (18.89 ± 8.87 *vs* 24.63 ± 3.52, $P < 0.05$). The nonarterial stenosis group showed higher preoperative popliteal artery (POA) blood flow than the arterial stenosis group (204.89 ± 80.76 cc/min *vs* 76.75 ± 48.49 cc/min, $P < 0.05$). Compared with the baseline (before surgery), the postoperative POA blood flow of the affected limb in the nonarterial stenosis group decreased one month after surgery (134.11 ± 47.84 cc/min *vs* 204.89 ± 80.76 cc/min, $P < 0.05$), while that in the arterial stenosis group increased (98.44 ± 30.73 cc/min *vs* 61.69 ± 21.70 cc/min, $P < 0.05$). Although the POA blood flow in the arterial stenosis group was obviously improved one month after surgery, it was still lower than that in the nonarterial stenosis group (98.44 ± 30.73 cc/min *vs* 134.11 ± 47.84 cc/min, $P < 0.05$). The nonarterial stenosis group had higher preoperative plantar microcirculation than the arterial stenosis group (56.1 ± 9.2 *vs* 33.2 ± 7.5, $P < 0.05$); compared with the baseline, the plantar microcirculation in the arterial stenosis group was significantly improved one month after surgery (51.9 ± 7.2, $P < 0.05$), while that in the nonarterial stenosis group was reduced (35.9 ± 7.2, $P < 0.05$).

CONCLUSION

Based on preoperative HFCDU findings, diabetic foot patients can be divided into two categories: Those with nonarterial stenosis and those with arterial stenosis, with obvious differences in hemodynamic changes in the early postoperative period between them. In the early stage after TTT, the blood flow volume and velocity and the plantar microcirculation perfusion of the affected limb of the diabetic foot with nonarterial stenosis decreased compared with the baseline, while those of the diabetic foot with arterial stenosis improved significantly compared with the baseline, although both had smoothly healed diabetic foot ulcers.

Key Words: High-frequency color Doppler ultrasonography; Diabetic foot; Tibial transverse transport; Nonarterial stenosis; Arterial stenosis

Core Tip: Diabetes foot can be categorized into arterial stenosis and non-arterial stenosis, which were significantly different in hemodynamics characteristics. This study tended to explore the hemodynamic findings and comparison of non-arterial stenosis group diabetic foot and arterial stenosis group diabetic foot after tibial transverse transport (TTT). In the early stage after TTT, the blood flow volume and velocity and the plantar microcirculation perfusion of the affected limb of the diabetic foot with nonarterial stenosis decreased compared with the baseline, while those of the diabetic foot with arterial stenosis improved significantly compared with the baseline, although both had smoothly healed diabetic foot ulcers.

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INTRODUCTION

Diabetic foot, a serious diabetes-related complication, has a high overall mortality rate of approximately 50% within 5 years[1]. At present, there are many methods to treat diabetic feet, with blood sugar control and local wound management as the primary treatment goals. For most patients with Wagner grade 1 to 2 diabetic foot ulcers, wound healing can be achieved by controlling blood sugar, dressing changes, negative pressure wound therapy, *etc*. However, for diabetic ulcers categorized as Wagner grade 3 and above, traditional treatment methods are less effective. In recent years, Chinese orthopedic surgeons have taken the lead in treating diabetic foot with the tibial transverse transport (TTT) technique, especially for cases graded as Wagner 3 and above, with excellent results achieved. Since 2019, our hospital has conducted clinical research on the treatment of diabetic foot with TTT and has also achieved encouraging effects.

During the treatment, we observed that the skin sensation and temperature of the affected foot were obviously improved, and the wounds healed smoothly after TTT therapy. Owing to its convenience, noninvasiveness and cost-effectiveness, high-frequency color Doppler ultrasonography (HFCDU) was used to dynamically observe the changes in blood circulation in the affected limb. It was found that diabetic foot can be divided into arterial stenosis and nonarterial stenosis categories that are significantly different in terms of hemodynamic characteristics. Here, we summarize and report our research results.

MATERIALS AND METHODS

Study subjects

Twenty-five patients with diabetic foot who underwent TTT in the Department of Orthopedics of Renmin Hospital of Wuhan University from January 2021 to August 2022 were selected. There were 20 males and 5 females aged 51 to 78 (mean: 63 ± 9.4) years, with Wagner grades 3, 4, and 5 confirmed in 4, 18, and 3 cases, respectively. Inclusion criteria: (1) Age: 18-80 years; (2) Wagner grade 3-5 diabetic foot; and (3) Good compliance and willingness to receive TTT therapy. Exclusion criteria: (1) > 75% stenosis in the lumen of the femoral popliteal artery (POA) on the affected side; (2) New cardio-cerebrovascular accidents within the last 3 mo; and (3) Inability to receive anesthetics due to contraindications to anesthesia. The Ethics Committee of Renmin Hospital of Wuhan University (Review Number: WDRY2022-K200) granted approval for this research, and informed consent was obtained from the subjects.

Instruments and methods

Ultrasonography: Instrument selection: A Philips EPIQ5 color ultrasonic diagnostic instrument was selected, with a linear array probe and a frequency range of 5.0-12 MHz. The common femoral artery (CFA), deep femoral artery, superficial femoral artery (SFA), POA, anterior tibial artery (ATA), posterior tibial artery (PTA), peroneal artery (PA), and dorsalis pedis artery were examined successively. The arterial inner diameter (ID), intima-media thickness (IMT), peak flow velocity (PFV), resistance index, and blood flow were measured. Atherosclerotic plaques of various blood vessels and medial arterial calcification (MAC) were observed. The lumen stenosis rate and the calcification rate were calculated, of which the stenosis rate was assessed by referring to the ultrasound criteria for the diagnosis of lower limb arterial disease established by Cossman *et al*[2]. Patients with ≥ 50% vascular stenosis in any of the femoral arteries, POA, PTA, ATA or PA of the affected limb were classified as the arterial stenosis group; otherwise, they were classified as the nonarterial stenosis group.

The score of femoral and popliteal atherosclerosis severity was based on less than 75% stenosis in the CFA, SFA or POA. The CFA, SFA and POA were scored according to the following criteria to assess the degree of femoral popliteal atherosclerosis. Scoring criteria: (1) IMT: Not thick (< 1 mm), 0 points; mild thickening (1-1.2 mm), 1 point; moderate and severe thickening (> 1.2 mm), 2 points; (2) Arterial plaques: 0 for normal (not found), 1 for single, 2 for multiple, and 3 for diffuse; and (3) Arterial stenosis: 0 for normal, 1 for 30%-49% stenosis, and 2 points for 50%-75% stenosis.

Below-knee artery outflow tract score: ATA, PTA, and PA were scored according to the outflow tract standard of the Society for Vascular Surgery[3]: ATA, PTA and PA were assigned scores according to the severity of the lesion, with 3 points for complete occlusion, 2.5 points for partial occlusion, 2 points for > 50% stenosis, 1 point for < 50% stenosis, and 0 for no stenosis. The outflow tract integral was the sum of the scores of these three arteries plus the base value of 1.

Lower-limb MAC score: Score was based on the length of the artery wall involved, with no calcification deposit, calcification range < 1/3, calcification range of 1/3-2/3, and calcification range > 2/3 being assigned 0, 1, 2, and 3 points, respectively. The MAC scores of the CFA, SFA, POA, ATA, PTA and PA were assessed, and the total score of each segment was summed. The POA blood flow and the ID and PFV of the ATA, PAT, PA and dorsalis pedis artery were measured.

Plantar microcirculation detection: PeriScanPIM3, a laser Doppler flowmeter system, was used to detect changes in plantar microcirculation before and 1 mo after surgery. The patient lay flat with the affected foot properly fixed, and a laser Doppler probe was placed on the sole to map plantar skin blood perfusion. Then, the anterior foot blood perfusion image was examined to determine the blood perfusion value of the corresponding area, which was the value of plantar microcirculation.

TTT: The operation was performed under nerve block or general anesthesia. After successful anesthesia, an about 10 cm arc incision was created in the medial middle of the tibia to separate the subcutaneous tissue of the periosteum. Then, the periosteum was cut along the medial tibia and peeled off to both sides of the tibia to determine the range of the bone window for tibial transport, which was 5 cm long and 1.5 cm wide. Next, two 2 mm external fixation screws were screwed into the bone window to move the bone block, followed by separation and removal of the bone block with a drill and a pendulum saw. During this process, attention was given not to damage the bone marrow in the medullary cavity, so that it formed a movable bone flap. Two external fixation screws with a diameter of 4 mm were then screwed into the proximal and distal tibia sides of the bone window, after which the lateral tibial transport frame was installed, adjusted and tightened, the bone transport direction was marked, and the periosteum, subcutaneous tissue and skin were closed layer by layer. During the operation, the ulcer lesions were thoroughly debrided, the abscess cavity was opened, the necrotic and inactivated skin and tendons were removed, the damaged tarsal bone and the blackened toe that lost blood supply were cut off, and the wound was bandaged with sterile dressing. The wound dressing was applied postoperatively. Bone transport was performed one week later, and wound healing was recorded.

Statistical methods

The data were analyzed by SPSS 22.0 statistical software. Continuous variables, described as the mean ± SD, were compared between groups by one-way ANOVA and within the same group before and one month after surgery by the paired *t* test. In all tests, a significance level of 5% ($P < 0.05$) was adopted.

RESULTS

Patient general information

All 25 patients underwent bone transport 1 wk after surgery, with the external fixator removed 4 wk later. The wound healing time of the diabetic foot was significantly shorter in the nonarterial stenosis group than in the arterial stenosis group (47.8 ± 13 *vs* 85.8 ± 26, P < 0.05), and the wound healing rate of both groups was 100%, as shown in Table 1.

HFCDU findings

The total lower extremity arteriopathy scores were markedly lower in the nonarterial stenosis group than those in the arterial stenosis group (18.89 ± 8.87 *vs* 24.63 ± 3.52, $P < 0.05$). In addition, the nonarterial stenosis group showed higher preoperative POA blood flow (cc/min) than the arterial stenosis group (204.89 ± 80.76 *vs* 76.75 ± 48.49, $P < 0.05$); one month after surgery, the POA blood flow (cc/min) was lower than the baseline in the nonarterial stenosis group (134.11 ± 47.84 *vs* 204.89 ± 80.76, $P < 0.05$), while the POA blood flow was higher than the baseline in the arterial stenosis group (98.44 ± 30.73 *vs* 76.75 ± 48.49, $P < 0.05$). One month after surgery, the POA blood flow in the arterial stenosis group was obviously improved, but it was still lower than that in the nonarterial stenosis group (98.44 ± 30.73 *vs* 134.11 ± 47.84, $P < 0.05$), as shown in Table 2.

Plantar microcirculation test results

The nonarterial stenosis group had better plantar microcirculation than the arterial stenosis group before surgery (56.1 ± 9.2 *vs* 33.2 ± 7.5, $P < 0.05$). One month after surgery, plantar microcirculation was significantly improved in the arterial stenosis group compared with the baseline (51.9 ± 7.2, $P < 0.05$), while it was reduced in the nonarterial stenosis group (35.9 ± 7.2, $P < 0.05$), as shown in Table 3. Typical cases are shown in Figures 1 and 2.

DISCUSSION

Improving the blood supply of the affected limb is a primary goal of TTT in treating diabetic feet

The development of the TTT technology is considered one of the milestones of orthopedic surgery in the 20th century and was originally created by Professor Ilizarov in accordance with the "Law of tension-stress" of tissue regeneration and the concept of "Natural Reconstruction". Scholars observed significant improvement in the blood circulation of the distal limbs of patients who underwent TTT[4], so they tried to use this technique to treat ischemic diseases of the lower limbs. In 2014, inspired by the use of TTT for the treatment of lower extremity ischemic disease, the team led by Professor Hua Qi-kai from the First Affiliated Hospital of Guangxi Medical University took the lead in attempting to treat diabetic feet using TTT, and the early clinical effect was satisfactory.

In TTT-related animal and clinical studies, scholars have confirmed through angiography and histology that in the process of bone transport, a large number of new blood vessels are generated around the transported bone block, including the end of the limb, constituting collateral circulation. In an animal experiment of TTT, Ilizarov[5,6] found that with longitudinal bone transport, damage to the bone marrow led to osteogenesis due to the influence of the lateral tensile stress vector, and capillaries, mainly continuous capillaries and sinusoidal capillaries, began to form 7 d after bone transport; the growth rate of blood vessels exceeded the transport rate on day 21 after bone transport, and the blood vessels became tortuous; angiography showed that a rich capillary network was formed around the bone transport block, and the density of blood vessels at the distal end of bone transport was also significantly increased. Cao *et al*[7] conducted experiments of TTT in dogs and confirmed that with the distraction of the external fixator, obvious microvascular regeneration could be seen in the bone transport area, extending distally along the trunk; moreover, both digital subtraction angiography and tissue hematoxylin and eosin staining confirmed the formation of a large number of microvascular networks around some of the main blood vessels. Through computed tomography angiography and perfusion imaging, some studies found that compared with preoperative patients, diabetic foot patients showed angiogenesis and increased blood perfusion within 3 mo after TTT[8,9], consistent with the growth of granulation tissue during ulcer healing[9]. These results are consistent with those observed in the arterial stenosis group in this study, suggesting that promoting angiogenesis and improving microcirculation in ischemic limbs or lesions is an important mechanism of TTT in the treatment of diabetic feet.

Characteristics and considerations of early hemodynamic changes in patients with nonarterial stenosis diabetic foot after TTT

In this study, there was no obvious stenosis in the main artery of the affected limb in the nonarterial stenosis group preoperatively and no obvious abnormalities in the POA blood flow volume or velocity despite abundant plantar microcirculation, but diabetic foot ulcers still persisted and did not heal, suggesting that vascular lesions or hemodynamic factors may not be the main cause of diabetic foot ulcers in such patients. B-ultrasound showed that the POA blood flow decreased one month after surgery compared with the baseline (before surgery), and correspondingly, the perfusion of plantar microcirculation decreased significantly. After TTT treatment, the granulation of the injured foot wound was fresh, the wound surface was obviously reduced, and finally, the ulcer healed smoothly, demonstrating that TTT can effectively treat diabetic foot ulcers without vascular stenosis. The significantly decreased rather than increased blood perfusion of the affected limb suggests that angiogenesis and increased blood perfusion may not be the main mechanism of TTT in the treatment of nondiabetic diabetic feet.

Table 1 General information

	Male/female	Age (yr)	Wagner grade				Non-arterial stenosis (n = 9)	Arterial stenosis (n = 16)
			3	4	5			
Patients (n = 25)	20/5	63.0 ± 9.4	4	18	3	The wound healing time (d)	47.8 ± 13	85.8 ± 26
						t value	4.090	
						P value	0.0004	

Table 2 Comparison of hemodynamics between the two groups

Group	Total lower extremity arteriopathy scores	POA blood flow (cc/min)	
		Preoperative	1 mo after surgery
Non-arterial stenosis (n = 9)	18.89 ± 8.87	204.89 ± 80.76	134.11 ± 47.84[a]
Arterial stenosis (n = 16)	24.63 ± 3.52	76.75 ± 48.49	98.44 ± 30.73[a]
t value	2.314	4.988	2.278
P value	0.030	< 0.0001	0.032

[a]P < 0.05 *vs* preoperative in the same group.
POA: Popliteal artery.

Table 3 Plantar microcirculation test results of the two groups

Group	Plantar microcirculation level	
	Before surgery	1 mo after surgery
Non-arterial stenosis (n = 9)	56.1 ± 9.2	35.9 ± 7.2[a]
Arterial stenosis (n = 16)	33.2 ± 7.5	51.9 ± 7.2[a]
t value	6.759	5.333
P value	< 0.0001	< 0.0001

[a]P < 0.05 *vs* preoperative in the same group.

Depending on etiology, diabetic foot ulcers can usually be divided into three types: Neuropathic, neuroischemic, and simple ischemic ulcers[10,11]. Most diabetic foot patients have neuropathy, and approximately 15% to 20% suffer from both neuropathy and peripheral artery diseases[12]. Neuropathy is one of the most common pathogenic factors of diabetic foot[11,13]. Pecoraro *et al*[12] attributed up to 82% of amputations in patients with diabetes to neuropathy, highlighting neuropathy as one of the main causes of diabetic foot ulcers. Diabetic neuropathy can involve motor, sensory, and autonomic nerve fibers, and its manifestation includes three symptoms. First, motor neuropathy causes muscle atrophy and weakness, resulting in an imbalance of strength in the foot flexor and extensor muscles. This imbalance can lead to a protrusion of the metatarsal bones and increased pressure on the local skin, resulting in a "claw toe" appearance. Second, sensory neuropathy causes a reduction in or loss of sensation, including pain, temperature, vibration and other stimuli, and repeated trauma to the feet, which contributes to ulcer formation[14]. Third, autonomic neuropathy induces autonomic nerve dysfunction, thereby impairing the sweating function of the lower limbs and causing dry and cracked skin, which makes the foot skin prone to repeated minor injuries[14]. Deformities and repeated injuries of the foot cause the skin of the foot to become swollen, predisposing it to ulcers.

In clinical practice, diabetic foot wounds recover normal skin coverage after TTT treatment instead of scar filling, suggesting that TTT may activate the tissue regeneration ability of the body through a certain mechanism that also facilitates skin regeneration[15]. In a study of TTT in the treatment of severe diabetic feet, the authors found that patients with diabetic foot experienced a significant reduction in numbness and improved skin sensation 2-3 mo after surgery and began to develop sensation after 6 mo, suggesting that nerve fibers may have regenerated in the affected limb during TTT treatment[16]. In related basic research, some researchers found that the local stretching effect of TTT can activate stem cells, improve peripheral blood levels of stromal cell-derived factor-1, vascular endothelial cell growth factor and other related factors, and regulate and mobilize the activation of stem cells. Moreover, it can improve the polarization of macrophages in distant wound tissue and balance the degree of inflammatory reaction by reducing the ratio of M1/M2 and regulating its composition to improve the "microenvironment" of wound healing and promote skin stem cells to

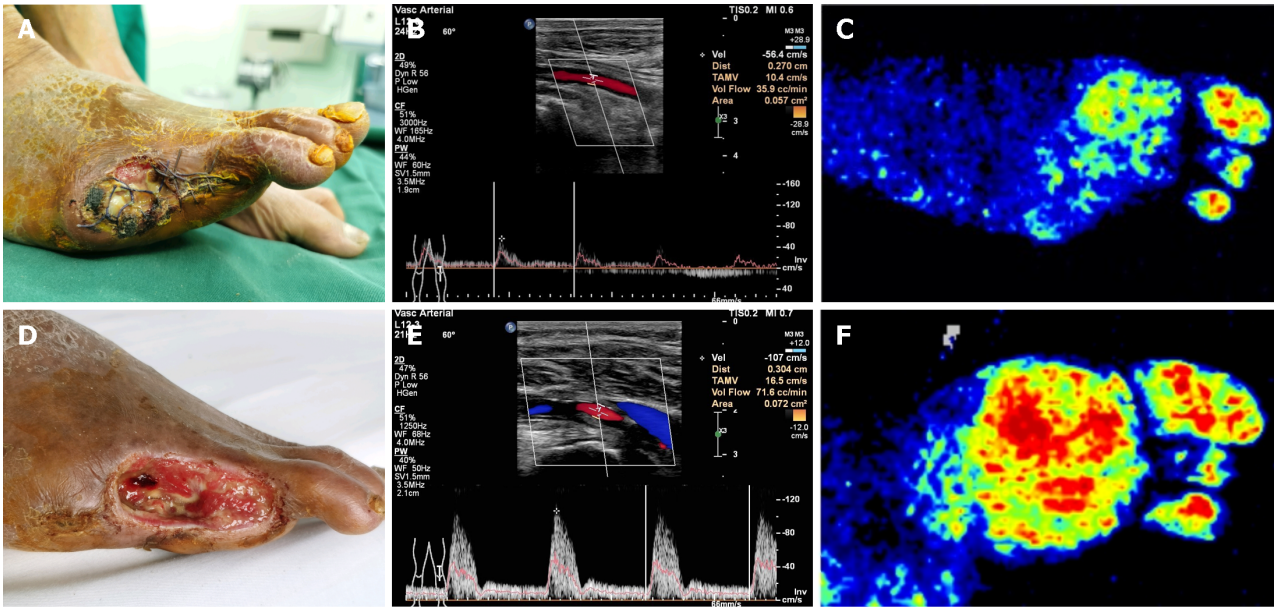

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Figure 1 With left diabetic foot and Wagner grade 4 foot ulcers. A: With left diabetic foot and Wagner grade 4 foot ulcers was amputated in a local hospital with non-healing chronic wounds and oozing; B and C: After referral to our department, the lower limb vascular score was 31 points and the popliteal artery (POA) was 36 cc/min (B) by preoperative B-ultrasound, with sparse plantar blood flow signals and poor microcirculation (C); D-F: One month after tibial transverse transport, the injured foot wound granulation was fresh and the wound was significantly reduced (D); B-ultrasound examination showed a significant increase in POA blood flow to 72 cc/min (E), abundant blood flow signals in plantar skin, and significant improvement in microcirculation (F).

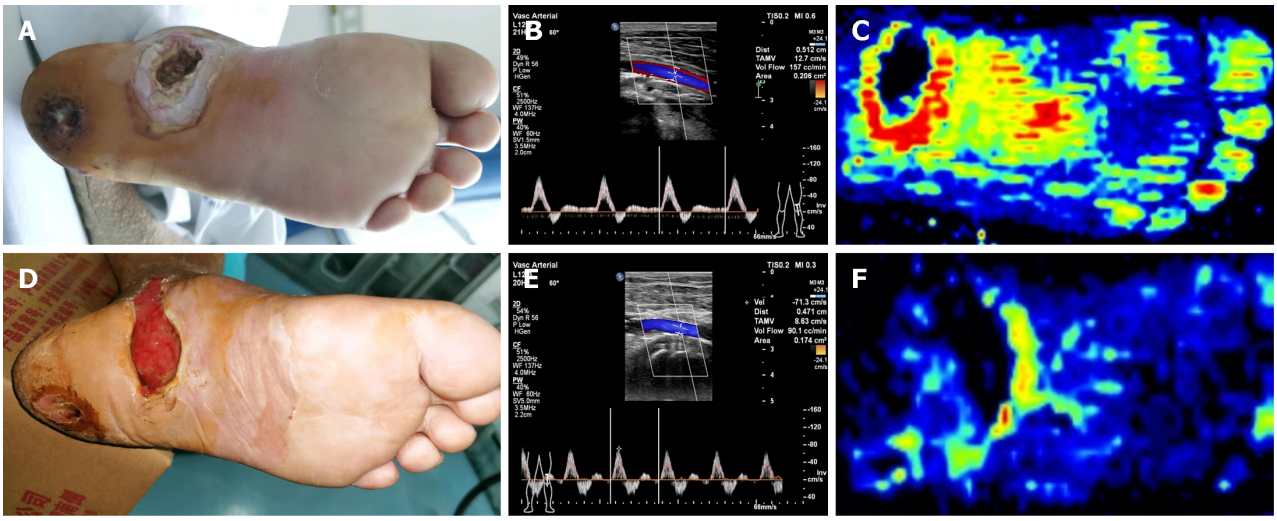

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Figure 2 Right diabetic foot of Wagner grade 3. A: Right diabetic foot of Wagner grade 3was treated by debridement and vacuum sealing drainage in the local hospital, with non-healing chronic wounds and oozing; B and C: After being referred to our department, the patient's lower extremity vascular score was 19 points during the preoperative B-ultrasound examination, the popliteal artery (POA) blood flow was 157 cc/min (B), the plantar blood flow signals were abundant, and the microcirculation was good (C); D-F: One month after tibial transverse transport, the wounds of the affected feet were fresh with granulation, and the wounds were obviously reduced (D); the POA blood flow decreased to 90 cc/min (E) by B-ultrasound, the plantar skin blood flow signals reduced, and the microcirculation decreased obviously (F).

participate in the regeneration and healing of ulcers[9,17]. Fos/Jun-related genes, FAK, the Wnt pathway and the Hippo pathway are activated during distraction osteogenesis, thus stimulating the regeneration potential of bone tissue. All the above pathways are closely associated with embryonic bone development or cell differentiation, suggesting that stimulating endogenous regeneration potential during embryonic development is the core principle of the Ilizarov technique for tissue regeneration[18]. Therefore, it can be inferred that TTT may help to regenerate tissue of the affected limb through some mechanism, and this regeneration may be include the vascular, nerve and skin systems, but further exploration regarding the regeneration mechanism is needed.

CONCLUSION

In clinical work, patients with diabetic foot can be divided into those with arterial stenosis and those with nonarterial stenosis according to the extent of stenosis of the femoral POA and three inferior genicular arteries on HFCDU images, and the hemodynamic changes in the early postoperative period between the two categories are obviously different. Compared with the preoperative conditions, the postoperative hemodynamic indices were significantly improved in patients with arterial stenosis and decreased in patients with nonarterial stenosis, although both had smoothly healed diabetic foot ulcers. It is suggested that TTT may have other possible mechanisms in addition to promoting angiogenesis and improving the microcirculation of the affected limb. TTT may achieve tissue regeneration of the affected limb through some mechanism, and this regeneration may be comprehensive, involving vascular, nerve, and skin regeneration. However, this regeneration mechanism needs to be further explored.

However, there are still some limitations in this study. First, this is a single-center, nonrandomized observational study with an insufficient sample size, thus further validation of the phenomena observed and the inferences expressed is warranted. Second, all 25 patients with diabetic foot were treated with TTT. Although encouraging results have been achieved, the choice of this method is largely based on the clinical experience of physicians, and no randomized controlled trials have been conducted. Finally, the pathological and molecular biological mechanisms of TTT in treating diabetic foot need further in-depth research.

ARTICLE HIGHLIGHTS

Research background

At present, there are many methods to treat diabetic feet, with blood sugar control and local wound management as the treatment principle. For most patients with Wagner grade 1 to 2 diabetic foot ulcers, wound healing can be achieved by controlling blood sugar, dressing change, negative pressure wound therapy, *etc.* But for Wagner grade 3 and above, the traditional treatment methods are less effective.

Research motivation

During the treatment, we observed that the skin sensation and temperature of the affected foot were obviously improved and the wounds healed smoothly after the tibial transverse transport (TTT) therapy. It was found that the diabetic foot can be divided into arterial stenosis and non-arterial stenosis categories that were significantly different in hemodynamic characteristics.

Research objectives

To evaluate the early hemodynamic changes in patients with non-arterial stenosis and arterial stenosis diabetic foot treated by TTT.

Research methods

Twenty-five patients with Wagner grade 3-5 diabetic foot ulcers were treated with TTT, and the wound healing time and rate were recorded. Patients were grouped according to the results of preoperative lower-extremity ultrasonography, classified as arterial stenosis group (n = 16); otherwise, they were classified as non-arterial stenosis group (n = 9). Before and one month after surgery, high-frequency color Doppler ultrasonography (HFCDU) was used to evaluate the degree of lower limb artery lesions and hemodynamic changes of patients. The degree of femoral-popliteal atherosclerotic stenosis, the degree of vascular stenosis and occlusion of the lower-knee outflow tract, and the degree of medial arterial calcification were scored; the three scores were added together to obtain the total score of lower extremity arteriopathy. Alterations in plantar microcirculation before and 1 mo after surgery were detected. Wound healing and hemodynamic indexes were compared between the two groups.

Research results

The wound healing time of diabetic foot was significantly shorter in non-arterial stenosis group than in arterial stenosis group, and the wound healing rate of both groups was 100%. Non-arterial stenosis group showed higher preoperative popliteal artery (POA) blood flow than arterial stenosis group. Although the POA blood flow in arterial stenosis group was obviously improved one month after surgery, it was still lower than that in non-arterial stenosis group. Non-arterial stenosis group had higher preoperative plantar microcirculation than arterial stenosis group.

Research conclusions

Patients with diabetic foot can be divided into arterial stenosis and non-arterial stenosis according to the stenosis of femoral POA and three inferior genicular arteries by HFCDU, and the hemodynamic changes in the early postoperative period between the two categories are obviously different.

Research perspectives

TTT may achieve tissue regeneration of the affected limb through some mechanism, and this regeneration may be comprehensive, involving vascular, nerve, and skin regeneration.

FOOTNOTES

Co-first authors: Mei-Mei Liao and Sen Chen.

Author contributions: Liao MM and Chen S contributed equally to this work and are co-first authors. Liao MM and Chen S contributed to the research design and thesis writing; Liao MM, Chen S, Cao JR, and Guo RQ collected and analyzed the data; Wang MW, Jin ZH, Ye J, and Ren YJ contributed to the data collection; Liao MM, Chen S, and Guo RQ overall supervise the study; and all authors contributed to the article and approved the submitted version.

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ORIGINAL ARTICLE

Retrospective Study

Establishment of models to predict factors influencing periodontitis in patients with type 2 diabetes mellitus

Hong-Miao Xu, Xuan-Jiang Shen, Jia Liu

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Abstract

BACKGROUND

Type 2 diabetes mellitus (T2DM) is associated with periodontitis. Currently, there are few studies proposing predictive models for periodontitis in patients with T2DM.

AIM

To determine the factors influencing periodontitis in patients with T2DM by constructing logistic regression and random forest models.

METHODS

In this a retrospective study, 300 patients with T2DM who were hospitalized at the First People's Hospital of Wenling from January 2022 to June 2022 were selected for inclusion, and their data were collected from hospital records. We used logistic regression to analyze factors associated with periodontitis in patients with T2DM, and random forest and logistic regression prediction models were established. The prediction efficiency of the models was compared using the area under the receiver operating characteristic curve (AUC).

RESULTS

Of 300 patients with T2DM, 224 had periodontitis, with an incidence of 74.67%. Logistic regression analysis showed that age [odds ratio (OR) = 1.047, 95% confidence interval (CI): 1.017-1.078], teeth brushing frequency (OR = 4.303, 95%CI: 2.154-8.599), education level (OR = 0.528, 95%CI: 0.348-0.800), glycosylated hemoglobin (HbA1c) (OR = 2.545, 95%CI: 1.770-3.661), total cholesterol (TC) (OR = 2.872, 95%CI: 1.725-4.781), and triglyceride (TG) (OR = 3.306, 95%CI: 1.019-10.723) influenced the occurrence of periodontitis ($P < 0.05$). The random forest model showed that the most influential variable was HbA1c followed by age, TC,

TG, education level, brushing frequency, and sex. Comparison of the prediction effects of the two models showed that in the training dataset, the AUC of the random forest model was higher than that of the logistic regression model (AUC = 1.000 *vs* AUC = 0.851; $P < 0.05$). In the validation dataset, there was no significant difference in AUC between the random forest and logistic regression models (AUC = 0.946 *vs* AUC = 0.915; $P > 0.05$).

CONCLUSION
Both random forest and logistic regression models have good predictive value and can accurately predict the risk of periodontitis in patients with T2DM.

Key Words: Type 2 diabetes mellitus; Periodontitis; Logistic regression; Prediction model; Random forest model; Gingival disease

Core Tip: With the rapid increase in the number of patients with type 2 diabetes mellitus (T2DM), the number of cases complicated by periodontitis has also increased. Without timely intervention, periodontitis can lead to tooth loosening and loss, and a decline in oral function, reducing patient quality of life. We retrospectively analyzed the data of 300 patients with T2DM to determine the factors influencing periodontitis. Random forest and logistic regression models were constructed to provide a theoretical basis for predicting periodontitis in patients with T2DM.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) accounts for more than 90% of cases of diabetes, and occurs mostly in adults over 40 years of age[1,2]. Research has shown that individuals with diabetes are more likely to develop periodontitis than those without[3]. Periodontitis is a chronic inflammation that occurs in periodontal supporting tissues and is characterized by gingival inflammation, formation of a periodontal pocket, resorption and destruction of the alveolar bone, and tooth loosening, displacement, and loss[4]. It may also affect masticatory function and nutritional intake[5]. Many studies have shown a bidirectional relationship between T2DM and periodontitis, and the incidence of periodontitis in patients with T2DM is approximately 2-3 times that of the general population[6]. Many studies have reported on the factors influencing periodontitis in patients with T2DM, but the majority use logistic regression analysis, which cannot intuitively present the importance of the outcome[7,8]. With the advent of the era of big data, predictive models have become useful in predicting the occurrence of diseases, but there are few relevant prediction models for periodontitis in patients with T2DM. Studies have shown that among the multiple machine learning models for predicting the risk of kidney disease in patients with T2DM, the random forest model has better performance and higher accuracy than logistic regression[9]. Therefore, the objective of this study was to retrospectively analyze the factors influencing periodontitis in patients with T2DM, and establish random forest and logistic regression prediction models for this disease.

MATERIALS AND METHODS

Subjects

Three hundred patients with T2DM who were hospitalized at the First People's Hospital of Wenling from January 2022 to June 2022 were selected as research subjects, and their relevant data were collected for this retrospective study (Figure 1). The inclusion criteria were: (1) Age ≥ 18 years old; and (2) T2DM diagnosed at our hospital and without other serious complications. The exclusion criteria were: (1) Patients with other systemic diseases affecting periodontal health; (2) Serious organ disorders; (3) Cognitive or mental disorders; (4) Periodontal treatment in the past 3 mo; (5) Pregnancy; (6) Unable to accept oral periodontal examination; and (7) Incomplete data.

Methods

General information and clinical examination data were collected from hospital records. General patient information included monthly income, age, education level, body mass index (BMI), sex, smoking, and alcohol intake. We also asked about daily brushing frequency and exercise habits. Clinical examination data included glycosylated hemoglobin (HbA1c), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels.

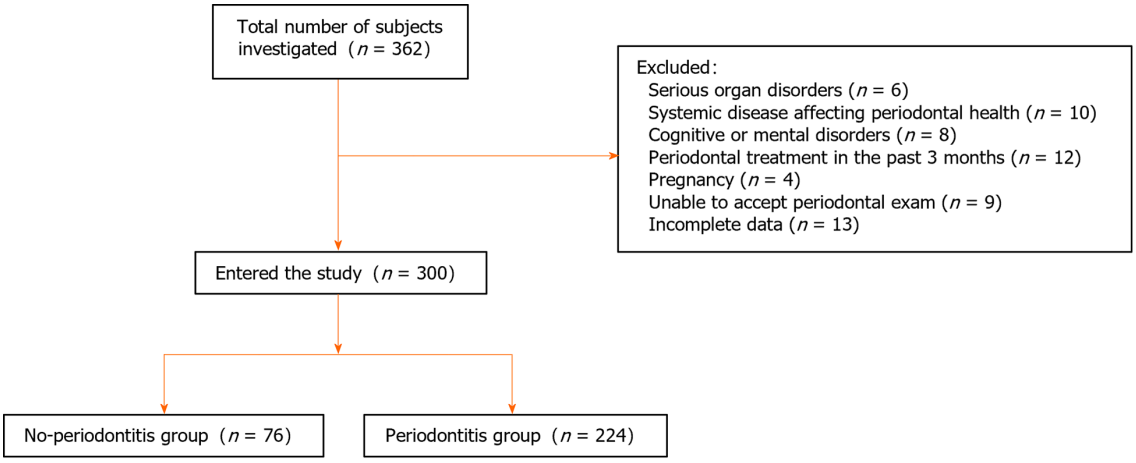

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Figure 1 Flow diagram.

Diagnostic criteria and classification of periodontitis

The Diagnostic criteria for periodontitis were: (1) A definite history of periodontitis; (2) Loose teeth in the mouth due to periodontitis without other factors; and (3) Periodontal examination showing that the depth of a periodontal pocket of at least one index tooth was ≥ 4 mm, and the tooth had clinical attachment loss (AL) ≥ 1 mm. According to the diagnostic criteria for periodontitis, the patients were divided into periodontitis and no periodontitis groups. Based on the depth of the periodontal pocket, AL, and alveolar bone resorption, periodontitis was divided into three categories: (1) Mild: Gingival inflammation and bleeding on probing, probing depth of periodontal pocket ≤ 4 mm, AL 1-2 mm, and alveolar bone resorption not more than one third of root length; (2) Moderate: Gingival inflammation, bleeding on probing or pus, depth of periodontal pocket ≤ 6 mm, AL 3-4 mm, alveolar bone resorption more than one third of the length of the root, but not more than half the length of the root, mild tooth loosening, and mild furcation lesions; and (3) Severe: Obvious inflammation accompanied by periodontal abscess, periodontal pocket depth > 6 mm, AL ≥ 5 mm, alveolar bone resorption more than half of the root length, multiple root furcation lesions, and tooth loosening and displacement.

Classification of indicators

According to BMI [weight (kg)/height (m^2)], the patients were classified as: Underweight: BMI < 18.5; normal weight: BMI 18.5-24.0; overweight: BMI 24.0-28; obesity: BMI ≥ 28. According to HbA1c, blood glucose control was classified as: Ideal control: HbA1c < 6.5%; good control: HbA1c 6.5%-7.5%; poor control: HbA1c 7.5%-8.5%; very poor control: HbA1c ≥ 8.5%. The normal levels of TC, TG, HDL-C, and LDL-C are < 5.18 mmol/L, < 1.7 mmol/L, ≥ 1.04 mmol/L, and ≤ 3.37 mmol/L, respectively.

Statistical analysis

Patient information was analyzed using SPSS 26.0. For univariate analysis, the data that passed the normality test, represented by the mean ± SD, were compared by the *t*-test. Data that failed the normality test, presented as median (M) and 25% and 75% percentiles (P25, P75), were compared by the rank sum test. Count data, denoted as *n* (%), were compared by the χ^2 test. Statistical significance was set at $P < 0.05$. The variables that were statistically significant in the univariate analysis were included in the logistic regression to analyze the factors influencing periodontitis in T2DM patients. Logistic regression and random forest prediction models were constructed using the R, and the receiver operating characteristic curves of the training dataset and validation dataset of the two models were drawn. The predictive efficacy of the models was compared according to the area under curve (AUC), and the AUC was compared using the Delong test.

RESULTS

Analysis of general information and periodontitis

Among the 300 patients with T2DM, 224 had periodontitis, with an incidence of 74.67%. Among them, 83 (37.05%) had mild, 78 (34.82%) had moderate, and 63 (28.13%) had severe periodontitis. One hundred and sixty-four (54.67%) were male and 136 (45.33%) were female, with an incidence based on sex of 58.04% and 44.74%, respectively. The average age was 60.91 ± 9.49 years in the periodontitis group and 54.11 ± 14.69 years in the no periodontitis group. The proportion of patients with education level below high school was highest (43.75%) in the periodontitis group. In terms of monthly income, the highest proportion of monthly income in the periodontitis group was between 2000 and 5000 yuan (44.20%). Univariate analysis showed that there were significant differences in sex, age, and education level between the periodontitis and non-periodontitis groups ($P < 0.05$), but there was no significant difference in monthly income ($P > 0.05$;

Table 1).

Analysis of lifestyle and periodontitis

Of the patients with periodontitis, 23.21% smoked, 17.86% drank alcohol, 51.34% exercised regularly, and 63.39% brushed their teeth less than twice a day. Of the patients without periodontitis, 13.16% smoked, 14.47% drank alcohol, 56.58% exercised regularly, and 27.63% brushed their teeth less than twice a day. Univariate analysis showed that there was a significant difference in brushing frequency between the periodontitis and non-periodontitis groups ($P < 0.05$), but there were no significant differences in smoking, drinking, or regular exercise ($P > 0.05$; Table 2).

Analysis of physical health status and periodontitis

Among the 300 patients with T2DM, the overall BMI was 24.96 (22.36, 26.81). The BMI of patients with periodontitis was overweight at 24.86 (22.13, 26.65), and the BMI of patients without periodontitis was overweight at 25.61 (23.26, 27.23). There were 72.77%, 64.29%, and 58.48% of patients in the periodontitis group with normal TG, TC, and HDL-C, respectively. These were lower than the proportions of patients without periodontitis with normal TG, TC, and HDL-C. All patients had normal levels of LDL-C. The periodontitis group had the highest proportion of patients with very poor blood glucose control at 37.50%. The proportion of patients in the non-periodontitis group with good blood glucose control was 40.79%. Univariate analysis showed that there were significant differences in TG, TC, and HbA1c between the two groups ($P < 0.05$), but there were no significant differences in BMI, LDL-C, or HDL-C ($P > 0.05$; Table 3).

Multivariate logistic regression analysis

Whether patients with T2DM developed periodontitis (no occurrence = 0, occurrence = 1) was used as the dependent variable, and statistically significant variables (sex, age, brushing frequency, education level, HbA1c, TC, and TG) were used as independent variables in the univariate analysis (Table 4). Logistic regression analysis showed that age, brushing frequency, education level, HbA1c, TC, and TG were factors influencing periodontitis in patients with T2DM ($P < 0.05$). Older age was associated with a higher risk of periodontal disease [odds ratio (OR) = 1.047, 95% confidence interval (CI): 1.017-1.078]. Brushing teeth < 2 times/d was also associated with a higher risk of periodontal disease (OR = 4.303, 95%CI: 2.154-8.599). The higher the education level, the lower the risk of periodontitis (OR = 0.528, 95%CI: 0.348-0.800). Higher HbA1c, TC, and TG levels were associated with a higher risk of periodontal disease (OR = 2.545, 95%CI: 1.770-3.661, OR = 2.872, 95%CI: 1.725-4.781, OR = 3.306, 95%CI: 1.019-10.723, respectively; Table 5).

Random forest model

According to the random number method, patients were randomly divided into the training dataset ($n = 200$) and validation dataset ($n = 100$) according to 2/3 and 1/3 of the total number of patients, respectively. The seven variables (age, brushing frequency, HbA1c, TC, education level, TG, and gender) that were statistically significant in the univariate analysis were included in the random forest model. As shown in Figure 2, the importance of variables influencing the occurrence of periodontitis in patients with T2DM was ranked as HbA1c, age, TC, TG, educational level, brushing frequency, and sex.

Comparison between the random forest and logistic regression models

In the training dataset, the overall efficacy of the random forest model in predicting periodontitis in patients with T2DM was higher than that of the logistic regression model. The AUC of the random forest model was significantly higher than that of the logistic regression model ($P < 0.05$; Figure 3A, Table 6). In the validation dataset, the overall performances of the random forest and logistic regression models were comparable and there was no significant difference in AUC between them ($P > 0.05$; Figure 3B, Table 7).

DISCUSSION

Periodontitis is internationally recognized as the sixth most common complication of diabetes. With an increase in the number of diabetic patients, the number of people with periodontitis in the diabetic population is also increasing[10-12]. The results of this study showed that among 300 patients with T2DM treated at our hospital, 74.67% had periodontitis, which is higher than that reported by de Miguel-Infante *et al*[13] (23.8%) and Hong *et al*[14] (43.7%). The differences in the incidence of periodontitis may be linked to population differences in different countries and regions or to the lifestyle of people in different regions. The higher incidence of periodontitis in patients with T2DM in this study may be due to the older age of the study population as a whole and the irreversible damage to the periodontal tissue caused by inflammation[15].

Our study found that periodontitis in patients with T2DM was associated with several factors. Logistic regression analysis showed that age, brushing frequency, education level, HbA1c, TC, and TG were the factors significantly influencing periodontitis in patients with T2DM. The importance of the variables was ranked by the random forest model as HbA1c, age, TC, TG, education level, brushing frequency, and sex. The results of the two models were similar, indicating that the prediction results were reliable.

We found that the greater the HbA1c level, the higher the risk of periodontitis (OR = 2.545, 95%CI: 1.770-3.661), which is similar to the results of Wu *et al*[16], Qureshi *et al*[17], and Dhir *et al*[18]. HbA1c is reflective of a patient's blood glucose control in the past 2-3 mo. Poor control of blood glucose can cause more severe periodontitis symptoms, while good blood glucose control can delay the progression of periodontitis[19,20]. We also found that the risk of periodontal disease

Table 1 General information

Factor	Total number (*n* = 300)	Periodontitis group (*n* = 224)	Non-periodontitis group (*n* = 76)	*t*/χ^2/*Z*	*P* value
Sex, *n* (%)				4.050	0.044
Male	164 (54.67)	130 (58.04)	34 (44.74)		
Female	136 (45.33)	94 (41.96)	42 (55.26)		
Age (yr), (mean ± SD)	59.18 ± 11.40	60.91 ± 9.49	54.11 ± 14.69	-4.646	< 0.001
Education level, *n* (%)				-3.626	< 0.001
Below high school education	118 (39.33)	98 (43.75)	20 (26.31)		
High school education	98 (32.67)	76 (33.93)	22 (28.95)		
Above high school education	84 (28.00)	50 (22.32)	34 (44.74)		
Monthly income, *n* (%)				-1.890	0.059
< 2000 yuan	65 (21.67)	54 (24.11)	11 (14.47)		
2000-5000 yuan	133 (44.33)	99 (44.20)	34 (44.74)		
> 5000 yuan	102 (34.00)	71 (31.70)	31 (40.79)		

Table 2 Lifestyle factors

Factor	Total (*n* = 300)	Periodontitis group (*n* = 224)	Non-periodontitis group (*n* = 76)	χ^2	*P* value
Smoking, *n* (%)				3.500	0.061
No	238 (79.33)	172 (76.79)	66 (86.84)		
Yes	62 (20.67)	52 (23.21)	10 (13.16)		
Alcohol consumption, *n* (%)				0.460	0.497
No	249 (83.00)	184 (82.14)	65 (85.53)		
Yes	51 (17.00)	40 (17.86)	11 (14.47)		
Regular exercise, *n* (%)					
No	142 (47.33)	109 (48.66)	33 (43.42)	0.625	0.429
Yes	158 (52.67)	115 (51.34)	43 (56.58)		
Brushing frequency, *n* (%)				29.248	< 0.001
≥ 2 times/d	137 (45.67)	82 (36.61)	55 (72.37)		
< 2 times/d	163 (54.33)	142 (63.39)	21 (27.63)		

increased with age (OR = 1.047, 95%CI: 1.017-1.078). This is similar to the results of a study by de Miguel-Infante *et al*[13]. Age may be associated with disease as with increasing age, there is generally a weakened immune system making tissues more susceptible to the invasion of anaerobic bacteria and aggravating susceptibility to periodontitis[21]. Blood lipid levels, particularly higher levels of TC and TG, were associated with an increased risk of periodontal disease. This is similar to the results obtained by Dhir *et al*[18] and Ding *et al*[22]. High blood lipid levels in patients with T2DM may increase the body's susceptibility to periodontitis by promoting the expression of inflammatory factors, increasing the oxidative stress response and lipid peroxidation, inhibiting bone formation, and promoting bone resorption[23,24]. Low education level was also associated with a higher risk of periodontitis which has also been reported by de Miguel-Infante *et al*[13] and Yamamoto *et al*[25]. People with lower education may have less exposure to information about oral health and less understanding about the risk of periodontitis with T2DM. Unsurprisingly, less frequent brushing was associated with a higher risk of disease which has also been reported by Hong *et al*[14] and Chang *et al*[26]. Brushing removes food residue and reduces the reproduction of bacteria in the mouth, thereby decreasing the likelihood of developing periodontitis[27].

The random forest model is widely used, and some studies have used it to predict the risk of nephropathy, peripheral neuropathy, and foot ulcers in patients with T2DM[9,28,29]. We found that the random forest model was significantly better than the logistic regression model for the validation but not the test dataset. Both models had good predictive value with AUCs of 0.946 and 0.915, respectively. The random forest model is an ensemble learning method based on decision trees. Its advantage is that it requires less data, and its modeling process is more convenient and faster than logistic regression. However, the sensitivity, specificity, accuracy, recall, precision, and AUC of the training dataset of the random

Table 3 Physical health status

Factor	Total number (*n* = 300)	Periodontitis group (*n* = 224)	Non-periodontitis group (*n* = 76)	*t*/*Z*	*P* value
BMI (kg/m^2), median (P25, P75)	24.96 (22.36, 26.81)	24.86 (22.13, 26.65)	25.61 (23.26, 27.23)	-1.223	0.221
TG (mmol/L)	1.51 ± 0.28	1.53 ± 0.28	1.44 ± 0.26	-2.678	0.008
< 1.7	226 (75.33)	163 (72.77)	63 (82.89)		
≥ 1.7	74 (26.67)	61 (27.23)	13 (17.11)		
TC (mmol/L)	4.72 ± 0.77	4.86 ± 0.78	4.33 ± 0.59	-5.361	< 0.001
< 5.18	212 (70.67)	144 (64.29)	68 (89.47)		
≥ 5.18	88 (29.33)	80 (35.71)	8 (10.53)		
HDL-C (mmol/L)	1.09 ± 0.19	1.08 ± 0.20	1.12 ± 0.15	1.777	0.077
< 1.04, *n* (%)	114 (38.00)	93 (41.52)	21 (27.63)		
≥ 1.04, *n* (%)	186 (62.00)	131 (58.48)	55 (72.37)		
LDL-C (mmol/L)	2.42 ± 0.29	2.44 ± 0.29	2.39 ± 0.30	-1.183	0.238
< 3.37, *n* (%)	300 (100.00)	224 (100.00)	76 (100.00)		
≥ 3.37, *n* (%)	0 (0.00)	0 (0.00)	0 (0.00)		
HbA1c (mmol/L)	7.86 ± 1.17	8.10 ± 1.16	7.14 ± 0.85	-6.596	< 0.001
< 6.5%, *n* (%)	37 (12.33)	17 (7.59)	20 (26.32)		
6.5%-7.5%, *n* (%)	83 (27.66)	52 (23.21)	31 (40.79)		
7.5%-8.5%, *n* (%)	90 (30.00)	71 (31.70)	19 (25.00)		
≥ 8.5%, *n* (%)	90 (30.00)	84 (37.50)	6 (7.89)		

BMI: Body mass index; TG: Triglyceride; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; HbA1c: Glycosylated hemoglobin.

Table 4 Assignment and description of related factors affecting the occurrence of periodontitis in patients with type 2 diabetes mellitus

Factor	Assignment of value
Sex	Female = 0
	Male = 1
Age	Continuous variables
Brushing frequency	≥ 2 times/d = 0
	< 2 times/d = 1
Education level	Below high school education = 0
	High school education = 1
	Above high school education = 2
HbA1c	Continuous variables
TC	Continuous variables
TG	Continuous variables

HbA1c: Glycosylated hemoglobin; TC: Total cholesterol; TG: Triglyceride.

Table 5 Multivariate logistic regression analysis of occurrence of periodontitis in patients with type 2 diabetes mellitus

Factor	β	SE	Wald χ^2	*P* value	OR (95%CI)
Age	0.046	0.015	9.813	0.002	1.047 (1.017-1.078)
Sex	0.622	0.346	3.230	0.072	1.863 (0.945-3.674)
Brushing frequency	1.459	0.353	17.073	< 0.001	4.303 (2.154-8.599)
Education level	-0.639	0.212	9.065	0.003	0.528 (0.348-0.800)
HbA1c	0.934	0.185	25.392	< 0.001	2.545 (1.770-3.661)
TC	1.055	0.260	16.451	< 0.001	2.872 (1.725-4.781)
TG	1.196	0.600	3.967	0.046	3.306 (1.019-10.723)

HbA1c: Glycosylated hemoglobin; TC: Total cholesterol; TG: Triglyceride; OR: Odds ratio; 95%CI: 95% confidence interval.

Table 6 Efficacy of the two models in predicting the occurrence of periodontitis in patients with type 2 diabetes mellitus in the training dataset

Model	Sensitivity	Specificity	Accuracy	Recall	Precision	AUC (95%CI)
Random forest	1.000	1.000	1.000	1.000	1.000	1.000 (1.000-1.000)
Logistic regression	0.569	0.919	0.830	0.569	0.707	0.851 (0.791-0.910)

AUC: Area under curve; CI: Confidence interval.

Table 7 Efficacy of the two models in predicting the occurrence of periodontitis in patients with type 2 diabetes mellitus in the validation dataset

Model	Sensitivity	Specificity	Accuracy	Recall	Precision	AUC (95%CI)
Random forest	0.520	0.960	0.850	0.520	0.813	0.946 (0.905-0.986)
Logistic regression	0.640	0.907	0.840	0.640	0.696	0.915 (0.857-0.973)

AUC: Area under curve; CI: Confidence interval.

forest model in this study reached 1, indicating that the model may have been overfitting. A logistic regression model is a commonly used probability prediction model, which is simple to use and has strong predictive ability. Its advantage lies in that it can quantify the risk of disease through the OR value of variables, but it cannot intuitively determine the importance of each independent variable to the model prediction.

This study has some limitations: (1) This was a retrospective study based on the data of patients diagnosed with T2DM at our hospital. The study subjects and sources are from one site and the sample size included in the model is small, so there is a certain sample bias; (2) This study analyzed only a subset of the factors that may influence periodontitis; and (3) The random forest model may have an issue with overfitting. Future studies should include samples from other regions and a more comprehensive analysis of factors that influence periodontitis, and construct a more complete prediction model.

CONCLUSION

In conclusion, the factors influencing periodontitis in patients with T2DM were identified using logistic regression analysis. In patients with T2DM, the greater the age and HbA1c, TC, and TG levels, the higher the risk of periodontitis. Our predictive models had good predictive value and could effectively predict the risk of periodontitis in patients with T2DM. The random forest and logistic regression prediction models can complement each other and provide a full analysis of the risk of disease and the importance of specific factors. In clinical practice, the results of this study can provide reference for the identification and intervention of early periodontitis in patients with T2DM.

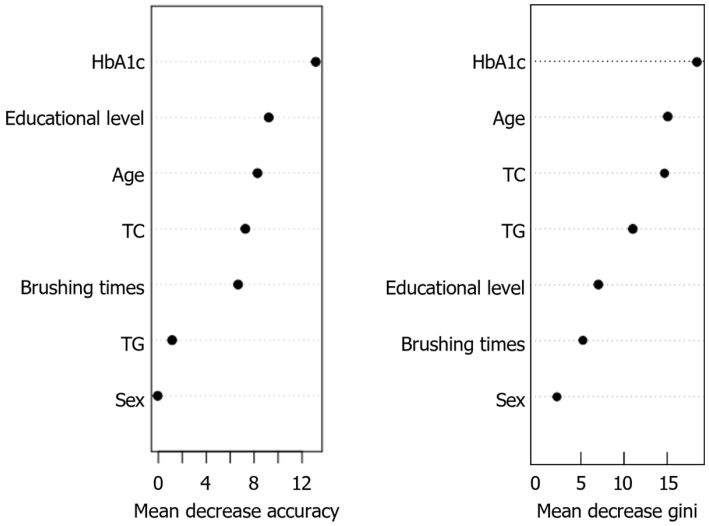

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Figure 2 Importance of variables in the random forest model. HbA1c: Glycosylated hemoglobin; TC: Total cholesterol; TG: Triglyceride.

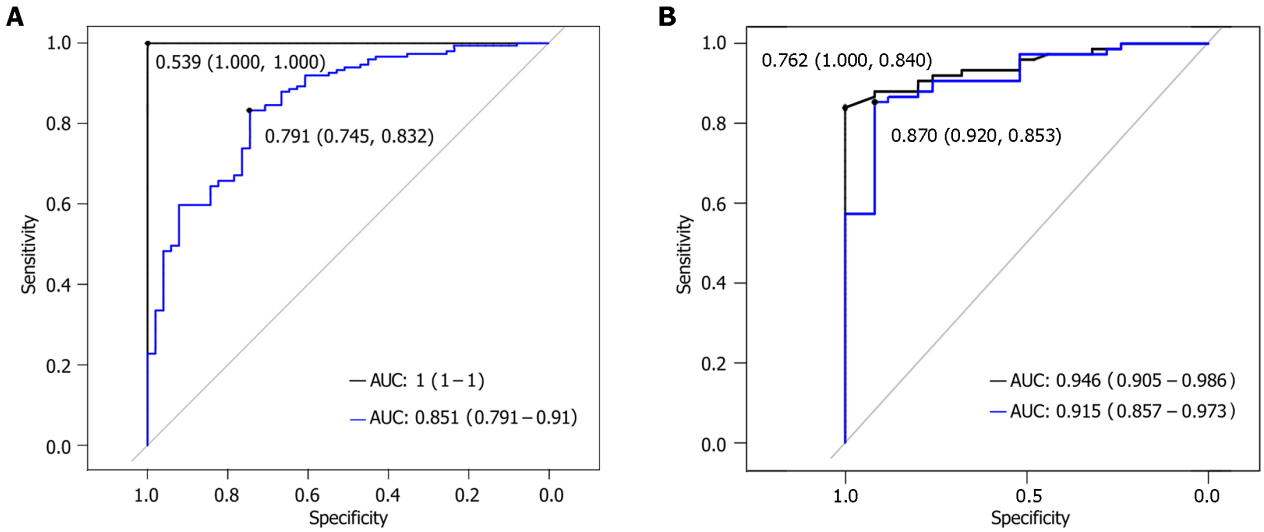

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Figure 3 Receiver operating characteristic curves of the random forest (black) and logistic regression (blue) models. A: For the training set; B: For the validation set. AUC: Area under curve.

ARTICLE HIGHLIGHTS

Research background

Periodontitis is a complication of type 2 diabetes mellitus (T2DM). With lifestyle changes and the acceleration of the aging process, the prevalence of periodontitis and diabetes is increasing annually.

Research motivation

Periodontitis can lead to tooth loosening and loss, decline in oral function, and reduced living standards.

Research objectives

This study aimed to explore and analyze the factors influencing periodontal disease in patients with T2DM, and construct prediction models for the risk of periodontal disease in patients with T2DM.

Research methods

We conducted a retrospective study in patients with T2DM hospitalized in our hospital to analyze the factors influencing periodontitis in patients with T2DM. We used random forest and logistic regression prediction models to assess the risk of specific factors in periodontitis.

Research results

This study found that the factors influencing periodontal disease in patients with T2DM were age, brushing frequency, education level, and glycosylated hemoglobin, total cholesterol, and triglyceride levels. The prediction models both had good predictive value.

Research conclusions

In this study, a random forest model was established and compared to a logistic regression model. The results showed that the random forest and logistic regression models had good predictive value and can accurately predict the risk of periodontitis in patients with T2DM.

Research perspectives

In the future, we will expand the sample size, combine samples from multiple regions, and include additional influencing factors to build a more complete prediction model.

FOOTNOTES

Author contributions: Xu HM designed the study and collected the data; Shen XJ analyzed the data; Liu J provided administrative support; and all authors have approved the manuscript.

Institutional review board statement: The study was reviewed and approved by the First People's Hospital of Wenling (approval No. KY-2023-2035-01).

Informed consent statement: Informed consent was waived due to the retrospective nature of this study.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The datasets used in this study can be obtained from the corresponding author upon reasonable request.

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Relationship between *GCKR* gene rs780094 polymorphism and type 2 diabetes with albuminuria

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Abstract

BACKGROUND

Diabetic kidney disease is one of the common complications of type 2 diabetes (T2D). There are no typical symptoms in the early stage, and the disease will progress to moderate and late stage when albuminuria reaches a high level. Treatment is difficult and the prognosis is poor. At present, the pathogenesis of diabetic kidney disease is still unclear, and it is believed that it is associated with genetic and environmental factors.

AIM

To explore the relationship between the glucokinase regulatory protein (*GCKR*) gene rs780094 polymorphism and T2D with albuminuria.

METHODS

We selected 252 patients (126 males and 126 females) with T2D admitted to our hospital from January 2020 to October 2020, and 66 healthy people (44 females and 22 males). According to the urinary albumin/creatinine ratio, the subjects were divided into group I (control), group II (T2D with normoalbuminuria), group III (T2D with microalbuminuria), and group IV (T2D with macroalbuminuria). Additionly, the subjects were divided into group M (normal group) or group N (albuminuria group) according to whether they developed albuminuria. We detected the *GCKR* gene rs780094 polymorphism (C/T) of all subjects, and measured the correlation between *GCKR* gene rs780094 polymorphism (C/T) and T2D with albuminuria.

RESULTS

Gene distribution and genotype distribution among groups I-IV accorded with the Hardy-Weinberg equilibrium. Genotype frequency was significantly different

among the four groups (P = 0.048, χ^2 = 7.906). T allele frequency in groups II, III, and IV was significantly higher than that in group I. Logistic regression analysis of the risk factors for T2D with albuminuria showed that the CT + TT genotype (odds ratio = 1.710, 95% confidence interval: 1.172-2.493) was a risk factor.

CONCLUSION
CT + TT genotype is a risk factor for T2D with albuminuria. In the future, we can assess the risk of individuals carrying susceptible genes to delay the onset of T2D.

Key Words: Type 2 diabetes mellitus; Albuminuria; Glucokinase regulatory protein rs780094; Gene polymorphism

Core Tip: Diabetic nephropathy (DN) is a serious complication of diabetes with no typical clinical manifestations at the beginning of the disease, and treatment efficacy is poor. Currently, it is believed that the pathogenesis of DN is associated with environmental and genetic factors. In this study, we found that CT + TT genotype in glucokinase regulatory protein rs780094 is a risk factor for type 2 diabetes complicated with albuminuria at the genetic level.

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INTRODUCTION

Type 2 diabetes (T2D) is a common chronic metabolic disease. The latest epidemiological survey showed an incidence rate of 10.3% for diabetes in China, of which T2D accounted for about 90%[1]. Diabetic nephropathy (DN) is one of the common complications of T2D. In China, the incidence rate of DN in patients with T2D is 20%-40%[2]. There are no typical symptoms in early kidney injury. When there is a high level of proteinuria and other symptoms, DN has reached the middle or late stage. At these stages, it is difficult to treat and often causes end-stage renal disease (ESRD), with a poor prognosis. Therefore, early and effective intervention in diabetes, regular monitoring of urinary protein, and timely symptomatic treatment can reduce the probability of T2D developing into DN and ESRD.

The pathogenesis of T2D and DN is not clear. Currently, it is believed to be caused by multiple factors. Genome-wide association study (GWAS) is a method of studying the association between a specific gene and a disease, using a large number of DNA samples for high density of single nucleotide polymorphisms genetic markers to find out the presence of sequence variations. Recent GWAS conducted domestically and internationally have identified > 250 candidate genes for susceptibility to T2D[3], such as *PRKAA2*[4], ATP binding cassette transporter 1[5], *FTO*[6], *FADS*[7], and glucokinase regulatory protein (*GCKR*)[8]. Human GCKR plays an important role in sugar regulation. At present, the genetic polymorphism of GCKR rs780094 is still controversial. Some studies believe that the T allele in GCKR rs780094 is related to the occurrence of T2D, and some scholars believe that the A allele is related to it. Because of the uncertainty of this relationship, it is worth further study.

MATERIALS AND METHODS

Research subjects

In this study, 252 T2D patients (126 males and 126 females) and 66 healthy people (44 females and 22 males) were selected by simple random sampling from January 2020 to October 2020 at our hospital. All subjects were free of acute infection and secondary diabetes (such as acromegaly or Cushing's syndrome), and were not pregnant. Patients with type 1 diabetes were excluded.

Patient grouping

According to the 1999 World Health Organization diagnostic criteria for T2D and the consensus of Chinese experts on prevention and treatment of diabetes in 2014, all subjects were divided into group I (control group), group II [diabetes with normoalbuminuria, urinary albumin/creatinine ratio (UACR) < 30 mg/mg], group III (diabetes with microalbuminuria group, 30-299 mg/mg), and group IV (diabetes with albuminuria, UACR ≥ 300 mg/mg). Additionally, the subjects were divided into either group M (normal group) or group N (albuminuria group). The study was approved by the Ethics Committee of the Affiliated Hospital of Southwest Medical University.

Questionnaire survey

All study populations used a unified survey questionnaire, which included name, gender, age, birth date, disease history, drug use, smoking history (never smoking refers to never smoking; smoking refers to still smoking in the past 30 d), and alcohol consumption (never drinking; occasional drinking < 1 time/wk in the past year; frequent drinking ≥ 1 time/wk in the past year).

Physical and biochemical examinations

We recorded the patients' height and weight and calculated their body mass index (BMI). Fasting blood was collected to detect 2-h postprandial blood glucose, fasting insulin, fasting C-peptide, blood lipid levels, *etc*. The glucose oxidase method was used for blood glucose detection; C-peptide and insulin were measured by radioimmunoassay; glycated hemoglobin was detected by hyphenated to liquid chromatography; and triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), blood urea nitrogen (BUN), and blood uric acid (BUA) were measured using a Hitachi 7600 automatic biochemical analyzer. The levels of urinary albumin and creatinine were detected with an automatic urine analyzer, and UACR was calculated. In addition, the subjects underwent oral glucose tolerance testing (OGTT).

DNA extraction and detection of gene polymorphism with TaqMan probe

TaqMan fluorescent probe is a kind of oligonucleotide probe. During polymerase chain reaction (PCR) amplification, a specific fluorescent probe is added along with a pair of primers. When the probe is complete, the fluorescence signal emitted by the reporter group is absorbed by the quencher group. During PCR amplification, the 5′-3′ exonuclease activity of Taq enzyme degrades the probe, separating the reporter fluorophores from the quench fluorophores, so that the fluorescence monitoring system can receive the fluorescence signal, that is, for each amplified DNA strand, a fluorescence molecule is formed, and the accumulation of fluorescence signal is completely synchronized with the formation of PCR products (Table 1 and Figure 1).

Statistical analysis

The research data were statistically analyzed using SPSS version 22.0. Measurement data with a normal distribution are expressed as the mean ± SD. Two independent samples *t*-test was used for comparison between two groups, and one-way analysis of variance was used for comparison among multiple groups. Measurement data with a non-normal distribution are expressed by median (interquartile interval). The Mann-Whitney *U* test was used for comparison between two groups. The Kruskal-Wallis *H* test was used for comparison among multiple groups. Numerical data were analyzed by the χ^2 test or Fisher's exact probability method. Multivariate logistic regression was used to analyze the influencing factors of T2D with albuminuria. $P < 0.05$ was considered statistically significant.

RESULTS

This study included 318 subjects, who were divided into group I (controls, $n = 66$), group II (diabetes with normoalbuminuria, $n = 101$), group III (diabetes with microalbuminuria, $n = 81$), and group IV (diabetes with macroalbuminuria, $n = 70$). Age, diastolic blood pressure, systolic blood pressure, weight, BMI, disease course, glycated hemoglobin, fasting blood glucose, 2-h postprandial blood glucose, BUN, BUA, creatinine, TG, TC, and UACR differed significantly among the groups ($P < 0.05$), while height, fasting insulin, fasting C-peptide, HDL, and LDL did not differ significantly ($P > 0.05$) (Table 2).

Some samples were selected for sequencing identification, and the sequencing results and probe results were completely consistent with the typing results (Figure 2). The genotype frequency and allele distribution of the control, normoalbuminuria, microalbuminuria, and macroalbuminuria groups are shown in Table 3. The gene distribution among the four groups and the whole genotype distribution were in accordance with the Hardy-Weinberg equilibrium ($P > 0.05$). The genotype frequency differed significantly among the four groups ($P = 0.048$, $\chi^2 = 7.906$). There were significant differences between the control and normoalbuminuria groups ($P = 0.012$, $U = 2613$), between the control and microalbuminuria groups ($P = 0.024$, $U = 2131$), and between the control and macroalbuminuria groups ($P = 0.027$, $U = 1836.5$). There were significant differences in genotype frequency among the four groups ($P = 0.032$, $\chi^2 = 8.786$). There were significant differences between the control and normoalbuminuria groups ($P = 0.007$, $U = 11328$), between the control and microalbuminuria groups ($P = 0.017$, $U = 9192$), between the control and macroalbuminuria groups ($P = 0.020$, $U = 7938$), and between the normoalbuminuria and microalbuminuria or macroalbuminuria groups. There was no significant difference in gene distribution or genotype distribution between the microalbuminuria and macroalbuminuria groups ($P > 0.05$) (Table 3).

T2D complicated with albuminuria was analyzed by logistic regression with diastolic blood pressure, systolic blood pressure, height, weight, BMI, disease course, glycated hemoglobin, fasting blood glucose, 2-h postprandial blood glucose, BUN, creatinine, TG, TC, UCAR as dependent variables, and each genotype as independent variables. Diastolic blood pressure, systolic blood pressure, weight, BMI, hypertension, hyperlipidemia history, history of alcohol consumption, glycated hemoglobin, fasting blood glucose, 2-h postprandial blood glucose, BUN, TG, TC, and CT + TT genotype were identified to be risk factors for T2D with albuminuria (Table 4).

Table 1 Probe sequence

Name	SNP site	Primer	Sequence	Modification	
				5'	3'
Human	rs780094	rs780094-F	GGCCCCAGTTTTTTAGACCAT		
		rs780094-R	GCCCGGCCTCAACAAAT		
		rs780094-PG	CTGACACATGTTTGCT	FAM	MGB
		rs780094-PA	TGACACATATTTGCTG	VIC	MGB

SNP: Single nucleotide polymorphism.

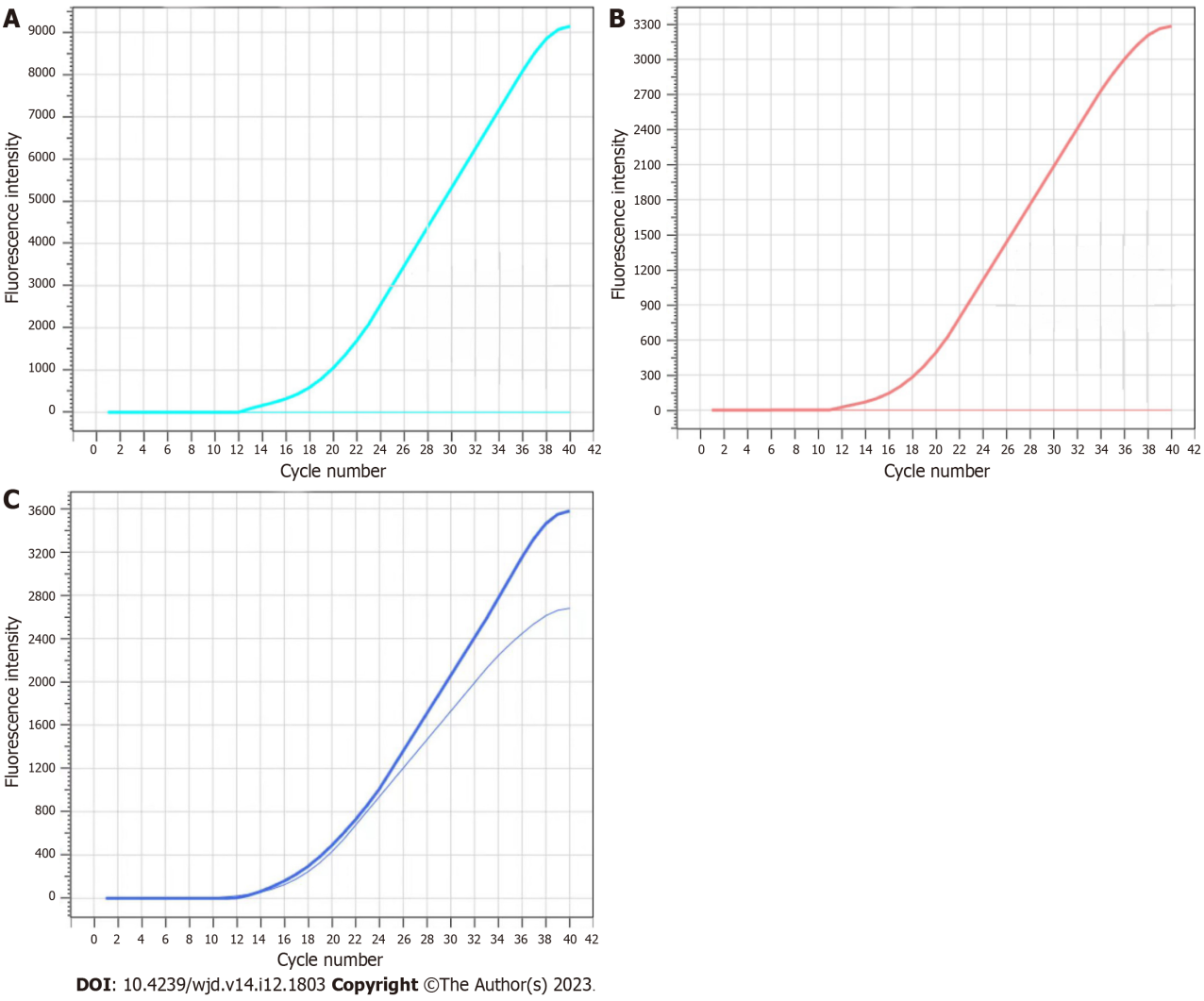

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Figure 1 Reaction diagram in a standard plasmid. A: rs780094-PA; B: rs780094-PG; C: rs780094-PA/G.

DISCUSSION

DN is one of the common complications of T2D and one of the main causes of ESRD[9]. At present, the pathogenesis of DN is not clear, and research shows that its pathogenesis is mainly related to long-term hyperglycemia, polyol pathway, microcirculatory disorder caused by oxidative stress, glycosylation of protein kinase, hyperfunction of platelet aggregation, increased glomerular filtration pressure, change of basement membrane charge, inflammatory reaction, and even dysbacteriosis[10,11]. However, these do not seem to fully explain the occurrence and development of DN. Therefore, it is increasingly believed that DN may be caused by environmental and genetic factors.

Glucokinase (GCK) is an important regulatory enzyme for glucose metabolism that can catalyze glucose phosphorylation in pancreatic islet β cells and mammalian liver cells, and it serves as a glucose sensor, regulating the function of pancreatic islets in releasing insulin and synthesizing glycogen. When glucose metabolism is normal, GCK binds to its

Table 2 Comparison of baseline data among the four groups

Group	I	II	III	IV	Statistics	*P* value
Number	66	101	81	70	-	-
Sex (male/female)	22/44	57/44[a]	36/45	33/37	8.755[1]	0.033
Age (yr)	51 (19)	55 (16)[a]	55.5 (15.75)[a]	58 (13.75)[a]	14.314[2]	0.003
DBP (mmHg)	73 (17.5)	87 (17.75)[a]	86 (17.75)[a]	92 (17.75)[a,b]	45.370[2]	< 0.001
SBP (mmHg)	125 (24.5)	153 (34.5)[a]	153.5 (35.5)[a]	154 (29)[a]	54.376[2]	< 0.001
Height (cm)	158 (11)	160 (14)	157 (10.75)	158.5 (14.75)	0.674[2]	0.879
Weight (kg)	54.4 (15.5)	62 (15.5)[a]	62 (13)[a]	64.5 (17.75)[a]	30.99[2]	< 0.01
BMI (kg/m^2)	21.74 (3.91)	25.3 (3.82)[a]	25.40 (4.53)[a]	25.39 (4.47)[a]	40.147[2]	< 0.01
Course of disease (mo)	-	90 (133.35)[a]	111.5 (132.5)[a]	118.5 (1332.5)[a]	147.932[2]	< 0.01
HbA1c (%)	5.7 (0.6)	9.6 (3.55)[a]	9.7 (3.5)[a]	9.35 (2.88)[a]	151.947[2]	< 0.01
FBG (mmol/L)	5.33 (0.75)	7.35 (2.93)[a]	8.85 (5.68)[a,b]	8.85 (3.2)[b]	106.139[2]	< 0.01
2-h PBG (mmol/L)	9.43 (1.83)	12.65 (2.45)[a]	13.4 (6.92)[a]	13.6 (7.47)[a,b]	102.209[2]	< 0.01
INS (mmol/L)	7.47 (5.79)	8.23 (8.54)[a]	8.0 (9.26)[a]	7.94 (8.01)	5.999[2]	0.112
Fasting C-peptide (mmol/L)	1.61 (1)	1.73 (1.58)	1.35 (1.62)	1.97 (2.37)	5.645[2]	0.130
BUN (mmol/L)	4.92 (1.79)	5.85 (2.15)[a]	5.67 (1.94)[a]	7.82 (2.56)[a,b,c]	56.728[2]	< 0.01
Cr (μmol/L)	55 (29.05)	58.6 (25.33)	57.4 (22.65)	87.95 (53.78)[a,b,c]	53.938[2]	< 0.01
UA (μmol/L)	347.6 (122.9)	318.25 (187.6)	298.95 (138.18)	389.1 (189.58)[b]	10.817[2]	0.013
TG (mmol/L)	1.42 (0.75)	1.69 (1.54)[a]	1.81 (1.40)[a]	1.74 (1.43)[a]	14.974[2]	0.02
TC (mmol/L)	3.77 (1.67)	4.51 (1.66)[a]	4.58 (1.53)[a]	4.44 (1.91)[a]	14.796[2]	0.02
HDL (mmol/L)	1.14 (0.35)	1.08 (0.40)	1.09 (0.30)	1.09 (0.40)	1.305[2]	0.728
LDL (mmol/L)	2.55 (1.14)	2.61 (1.45)	2.54 (1.42)	2.69 (1.95)	1.145[2]	0.766
UCAR (μg/mg)	18.95 (11.92)	21.9 (48.63)[a]	67.85 (67.3)[a,b]	2314.5 (3161.08)[a,b,c]	218.326[2]	< 0.01
Hypertension (yes/no)	57/9	64/37[a]	29/52[a,b]	31/39[a,b]	44.444[1]	< 0.01
Hyperlipidemia (yes/no)	52/14	62/39[a]	48/33[a]	46/24	7.301[1]	0.063
CHD (yes/no)	63/3	90/11	72/9	66/4	3.482[1]	0.323
Stoke (yes/no)	63/3	95/6	72/9	63/7	4.182[1]	0.242
Drink (yes/no)	55/11	66/35	60/21	45/35	8.359[1]	0.039
Smoke (yes/no)	53/13	68/33[a]	57/24	46/24[a]	4.305[1]	0.230

[a]Represents a statistically significant difference from group I.
[b]Represents a statistically significant difference from group II.
[c]Represents a statistically significant difference from group III.
[1]Represents χ^2 value.
[2]Represents *H* value.
DBP: Diastolic blood pressure; SBP: Systolic blood pressure; BMI: Body mass index; HbA1c: Glycosylated hemoglobin; FBG: Fasting blood glucose; 2-h PBG: 2-h postprandial blood glucose; BUN: Blood urea nitrogen; UA: Uric acid; TG: Triglyceride; TC: Total cholesterol; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; UCAR: Urinary albumin/creatinine ratio; CHD: Coronary heart disease.

inhibitory protein GCKR in the liver cell nucleus, causing an increase in glucose concentration, leading to dissociation of the GCK-GCKR complex, and promoting GCK translocation to the cytoplasm, glucose phosphorylation in liver cells, and insulin release and glycogen synthesis by pancreatic islet β cells[12], and GCKR transforms into inactive GCKR. rs780094 is a single-nucleotide polymorphism site in the noncoding region of the *GCKR* gene. It was first reported in a GWAS of T2D in 2007[13]. It was found that the *GCKR* gene was closely related to blood lipids in the Danish population, and that the level of TG in G allele carriers was reduced, accompanied by an increase in fasting plasma glucose. The insulin level assessed by the steady-state model was reduced, and insulin release related to OGTT was increased, slightly increasing the risk of T2D[14]. Subsequent in-depth analysis by GWAS showed that *GCKR* rs780094 was closely related to T2D and its complications. Zhou *et al*[15] found that carriers of the GCKR rs780094 C allele had a significantly higher risk of T2D. This conclusion is consistent with the large-scale meta-analysis conducted by Wang *et al*[16], which showed that *GCKR*

Table 3 Comparison of genotype frequency and allele frequency among the four groups

Group	*n*	CC	CT	TT	C	T
I	66	25 (37.9%)	28 (42.4%)	13 (19.7%)	78 (59.1%)	54 (40.9%)
II	101	22 (21.7%)	45 (44.6%)	34 (33.7%)	89 (44.1%)	113 (55.9%)
III	81	18 (22.2%)	37 (45.7%)	26 (32.1%)	73 (45.1%)	89 (54.9%)
IV	70	15 (21.4%)	33 (47.1%)	22 (31.5%)	63 (45.0%)	77 (55.0%)

Table 4 Logistic regression analysis of risk factors for type 2 diabetes mellitus complicated with proteinuria

Variable	B	SE	Wald χ^2	*P* value	OR (95%CI)
DBP (mmHg)	0.077	0.013	35.65	< 0.01	1.080 (1.053-1.017)
SBP (mmHg)	0.036	0.006	36.858	< 0.01	1.037 (1.025-1.049)
Weight (kg)	0.072	0.015	23.121	< 0.01	1.075 (1.044-1.107)
BMI (kg/m^2)	0.300	0.051	34.472	< 0.01	1.350 (1.221-1.492)
Hypertension (yes/no)	1.878	0.380	24.391	< 0.01	6.538 (3.103-13.773)
Hyperlipidemia (yes/no)	0.827	0.328	6.358	0.012	2.286 (1.202-4.346)
Drink (yes/no)	0.862	0.357	5.841	0.016	2.368 (1.177-4.766)
HbA1c (%)	4.834	0.904	28.614	< 0.01	125.687 (21.385-738.706)
FBG (mmol/L)	1.258	0.187	45.233	< 0.01	3.517 (2.438-5.074)
2-h PBG (mmol/L)	0.631	0.092	47.502	< 0.01	1.879 (1.571-2.248)
BUN (mmol/L)	0.477	0.099	23.410	< 0.01	1.612 (1.328-1.956)
TG (mmol/L)	0.464	0.159	8.548	0.003	1.591 (1.165-2.171)
TC (mmol/L)	0.470	0.122	14.801	< 0.01	1.600 (1.259-2.032)
CT + TT	0.536	0.192	7.765	0.005	1.710 (1.172-2.493)

OR: Odds ratio; CI: Confidence interval; DBP: Diastolic blood pressure; SBP: Systolic blood pressure; BMI: Body mass index; TG: Triglyceride; TC: Total cholesterol; 2-h PBG: 2-h postprandial blood glucose; HbA1c: Glycosylated hemoglobin; FBG: Fasting blood glucose; BUN: Blood urea nitrogen.

rs780094 mutation leads to an increased risk of cross-ethnic T2D. A study on Han Chinese showed a significant correlation between rs780094 and T2D[17]. Some studies have shown that *GCKR* is an independent susceptibility gene for T2D, and its T allele can reduce fasting blood glucose and the incidence rate of T2D[18]. Some studies have also shown that the incidence of T2D was reduced by the GCKR rs780094 G allele[19]. Li *et al*[20] and Bi *et al*[21] found racial differences in this effect. A study in the Han Chinese population showed that the A allele in GCKR rs780094 was associated with a reduced risk of T2D and obesity[22]. Another study showed that the GCKR rs780094 polymorphism was not associated with the occurrence of T2D[23]. We found that there was a significant difference in genotype frequency among groups I-IV, indicating that the differences in *GCKR* rs780094 in the population were related to glucose metabolism. This correlation is related to GCK as the first rate-limiting enzyme of the glucose metabolic pathway. This difference existed in the control group and T2D patients, but was not related to whether the patients had albuminuria, nor to the severity of albuminuria in the patients. It is speculated that the change from C to T can cause the substitution of an amino acid, thus affecting the activity of GCKR, but how GCKR acts on urinary protein warrants further study. Of course, it may also be related to the small sample size of our study and the variation of gene frequencies in different races, which still needs to be further explored by large-scale cohort studies in the future.

In our study, we also found that GCKR rs780094 was associated with type 2 diabetes mellitus, and this association was related to lipid levels. The possible reason is that obesity can release a large number of pro-inflammatory factors, which can increase the body's resistance to insulin. At the same time, these inflammatory factors can also interfere with the regulation of gene expression and the interaction between genes, thus affecting our glycolysis pathway and causing glucose metabolism disorders.

We also carried out a logistic correlation analysis on the factors related to T2D with albuminuria, and found that TG, TC, and CT + TT genotypes were risk factors. After adjusting blood pressure, BMI, and other indicators, the correlation was still significant. However, this significance was only expressed in the CC + CT genotype. We did not find this correlation in C, T, CC, CT, and TT genotypes. This may be due to the increased expression of GCKR accompanied by insulin resistance, and high insulin levels may stimulate the brush border of the proximal convoluted tubules, promote the exchange of UA and sodium ions, increase UA reabsorption, and thus increase UA levels[24]. The increase in UA level

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Figure 2 Sequencing maps. A: Patient with normoalbuminuria; B: Patient with microalbuminuria group; C: Patient with macroalbuminuria.

can damage the kidneys through a series of events, such as inflammatory reaction, destruction of endothelial cells, activation of the renin-angiotensin-aldosterone system, proliferation of vascular smooth muscle cells, causing renal vasoconstriction and thickening of glomerular arterial wall[25], and then production of albuminuria. Present and previous studies have shown that *GCKR* rs780094 is associated with T2D and T2D with albuminuria, and this correlation is related to UA, gender, and blood lipid level.

This study had some limitations. First, the sample size was small. Second, the selected subjects were from the Southwest region, which is geographically limited, so extrapolation of our results to other ethnic groups or the whole country should be cautious. Third, the effect of drugs on albuminuria was ignored. Finally, since we only selected the *GCKR* rs780094 locus for study, we may have ignored the impact of other gene polymorphisms on T2D with albuminuria. In future research, the sample size should be increased to conduct large-scale, multi-regional, and gene-locus-centered studies.

CONCLUSION

T2D and DN are the results of a variety of factors and their interactions, including environment, eating habits, lifestyle, race, and family history. Genetic factors also play an important role in the occurrence of diabetes. This is why a susceptible gene may exhibit different phenotypes in different populations or regions. Various studies have reported the relationship between genetic variation and susceptibility to T2D. In clinical practice, we can start with proteinuria detection, assess the risk of individuals carrying susceptibility genes, and take comprehensive prevention and control measures to delay the onset of T2D.

ARTICLE HIGHLIGHTS

Research background

Diabetic nephropathy (DN) is a serious complication of diabetes with no typical clinical manifestations at the beginning of the disease, and treatment efficacy is poor. Currently, it is believed that the pathogenesis of DN is associated with environmental and genetic factors. In this study, we found that CT + TT genotype in glucokinase regulatory protein (*GCKR*) rs780094 is a risk factor for type 2 diabetes (T2D) complicated with albuminuria.

Research motivation

Human GCKR plays an important role in sugar regulation. However, the association between *GCKR* gene rs780094 polymorphism and diabetes and its complications is uncertain.

Research objectives

To explore the relationship between the *GCKR* gene rs780094 polymorphism and T2D with albuminuria.

Research methods

The correlation between *GCKR* rs780094 and diabetes mellitus with proteinuria was studied by different grouping methods.

Research results

Studies have found that there are many risk factors for T2D with albuminuria. From the perspective of environmental factors, there were history of hypertension, alcohol consumption, history of hyperlipidemia, and blood glucose levels. At the genetic level, CT + TT genotype was identified to be a risk factor for T2D mellitus with albuminuria.

Research conclusions

In clinical practice, we can start with proteinuria detection, assess the risk of individuals carrying susceptibility genes, and take comprehensive prevention and control measures to delay the onset of T2D.

Research perspectives

While promising, the study has some limitations, including that it did not take into account whether patients were taking lipid-lowering and blood-pressure medications, and did not calculate insulin resistance indexes, among others. In addition, due to the limited geographical options in this study, there may be selection bias, and further clinical trials are needed to refine the conclusions of this study.

FOOTNOTES

Author contributions: Liu YY was responsible for experimental design and implementation, and paper writing; Wan Q was responsible for quality review and control.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Acupuncture in diabetic peripheral neuropathy-neurological outcomes of the randomized acupuncture in diabetic peripheral neuropathy trial

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Abstract

BACKGROUND

Diabetic peripheral neuropathy (DPN) is a common complication of diabetes mellitus and can lead to serious complications. Therapeutic strategies for pain control are available but there are few approaches that influence neurological deficits such as numbness.

AIM

To investigate the effectiveness of acupuncture on improving neurological deficits in patients suffering from type 2 DPN.

METHODS

The acupuncture in DPN (ACUDPN) study was a two-armed, randomized, controlled, parallel group, open, multicenter clinical trial. Patients were randomized in a 1:1 ratio into two groups: The acupuncture group received 12 acupuncture treatments over 8 wk, and the control group was on a waiting list during the first 16 wk, before it received the same treatment as the other group. Both groups received routine care. Outcome parameters were evaluated after 8, 16 and 24 wk and included neurological scores, such as an 11-point numeric rating scale (NRS) 11 for hypesthesia, neuropathic pain symptom inventory (NPSI), neuropathy deficit score (NDS), neuropathy symptom score (NSS); nerve

conduction studies (NCS) were assessed with a handheld point-of-care device.

RESULTS

Sixty-two participants were included. The NRS for numbness showed a difference of 2.3 ($P < 0.001$) in favor of the acupuncture group, the effect persisted until week 16 with a difference of 2.2 ($P < 0.001$) between groups and 1.8 points at week 24 compared to baseline. The NPSI was improved in the acupuncture group by 12.6 points ($P < 0.001$) at week 8, the NSS score at week 8 with a difference of 1.3 ($P < 0.001$); the NDS and the TNSc score improved for the acupuncture group in week 8, with a difference of 2.0 points ($P < 0.001$) compared to the control group. Effects were persistent in week 16 with a difference of 1.8 points ($P < 0.05$). The NCS showed no meaningful changes. In both groups only minor side effects were reported.

CONCLUSION

Study results suggest that acupuncture may be beneficial in type 2 diabetic DPN and seems to lead to a reduction in neurological deficits. No serious adverse events were recorded and the adherence to treatment was high. Confirmatory randomized sham-controlled clinical studies with adequate patient numbers are needed to confirm the results.

Key Words: Diabetic peripheral neuropathy; Numbness; Nerve conduction study; Acupuncture

Core Tip: Diabetic peripheral neuropathy affects a high number of diabetic patients. It can lead to painful sensations of the lower extremities and loss of sensory function. The latter can lead to gait instability, falls and injury of the feet. Pharmacological treatments can only reduce painful symptoms but do not improve numbness; furthermore, they add to pharmacological burden of multimorbid patients. Acupuncture is a safe option to treat chronic pain; the potential to improve sensory loss is evaluated in this trial.

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INTRODUCTION

According to the International Diabetes Federation (IDF), 537 million adults worldwide were already living with diabetes mellitus (DM) in 2021 and IDF estimates that there will be 783 million adults with diabetes by 2045[1]. Diabetic distal sensorimotor polyneuropathy occurs in approximately 28% of hospitalized diabetic patients and in those in primary care [2], it is the most common neurological complication of DM. Moreover, diabetic peripheral neuropathy (DPN) can already occur in the presence of impaired glucose tolerance and thus before the manifestation of diabetes[3]. DPN presents with stocking-like numbness, thermoanesthesia, hypesthesia, painful tingling, pricking, or burning sensations, and loss of proprioception. Especially hypesthesia and loss of proprioception may result in gait instability and falls; plantar injuries may go unnoticed and increase the risk for ulcerations or even amputation[4-6].

Pharmacological options to treat hypesthesia are missing. Symptomatic therapy addresses only plus-symptoms such as pain or tingling; here anticonvulsants and antidepressants are used[7]. According to a Cochrane meta-analysis even strict glycemic control showed no recovery or prevention of neuronal demise[8] and the used anticonvulsants have common side effects like fatigue or interact with other medication. Previous studies have also shown that the adherence of patients with neuropathic pain to the standard dosage of the above-mentioned medications is poor; this has been explained by the fear of side effects or the inadequate control of pain[9-11]. Non-pharmacological options for treatment are therefore of interest.

A meta-analysis in 2017 showed that acupuncture is an effective and safe treatment for chronic pain[12]. Besides pain control, several trials suggest that acupuncture also has a positive influence on nerve function with an improvement of nerve conduction[13-18].

The acupuncture in DPN (ACUDPN) trial investigates the effectiveness of acupuncture in diabetic patients suffering from DPN with a follow up of the effects until week 24. The results of the primary subjective parameters were published elsewhere and showed that acupuncture may be beneficial with a significant and clinically relevant reduction of overall DPN-related complaints and pain and disease-specific quality of life[19]. In this manuscript we focus on the effects of acupuncture on neurological examination scores and nerve conduction studies (NCS).

MATERIALS AND METHODS

The study protocol was approved by the ethics committee Berlin (EA1/183/18) and Hamburg, Germany in October 2018. ACUDPN was performed in compliance with the Declaration of Helsinki and standards of Good Clinical Practice. Informed written and oral consent was given by all patients prior to beginning of the study. The trial was registered on ClinicalTrials.gov NCT03755960.

This trial is a two-armed, randomized, controlled, parallel group, multicenter clinical trial. It was conducted between February 2019 and April 2021 at the German Charité Universitätsmedizin Berlin and at an outpatient clinic for TCM (HanseMerkur Center for TCM) at the University Medical Center Hamburg-Eppendorf in Hamburg, Germany. Details regarding study methodology have been published elsewhere[20].

Eligibility criteria

Participants had to meet the following criteria to be eligible for the study: Female or male aged 18-80 years; with a diagnosis of DPN with at least moderate symptoms and a minimum of at least 40 mm on a 0-100 mm visual analogue scale (VAS) for overall DPN-related complaints; pathological nerve conduction velocity (NCV) < 42 m/s and/or an amplitude of the sural nerve < 6 μV; absence of severe DPN with muscular weakness of the proximal leg muscles or neuropathy due to other reasons (such as borrelia infection, human immunodeficiency virus infection, hereditary factors, alcohol, or a history of neurotoxic drug use or traumatic lesions of the nerves or vessels in the lower extremities); body mass index < 35; absence of anticoagulation or bleeding disorders; absence of severe peripheral artery disease in Fontaine stage IV or ulcers or gangrenous lesions of the feet; severe fatigue syndrome; if needed then previously (since 4 wk) unchanged doses of pain medication against DPN; no opioid use before inclusion in the study or regular use of cannabis or cannabinoids or lipoic acid infusions planned during participation in the trial; no scheduled psychotherapy during study participation; no additional therapy with complementary medicine or physical therapy for symptoms of DPN during the 6 wk before inclusion in the study or planned during the study; no pregnancy or lactation. In addition, patients had to be able to complete a diary for the self-evaluation of symptoms, to record the use of symptomatic medication.

Randomization with a 1:1 ratio was performed using a computer-generated randomization list with the statistical package SAS (SAS 9.4, SAS Institute Inc., Cary, NC, United States). The study physician received the information about the allocation by phone from the study nurse.

All patients were enrolled in the trial for 24 wk. The patients in the intervention group received a total of 12 acupuncture sessions over the first 8 wk, the control group was on a waiting list and received the same acupuncture treatment from week 16 onwards. All patients kept diaries for the first 8 wk, completed questionnaires, and attended follow ups. Both groups were allowed to continue their usual medication during the study. A detailed description of the methodology has been published elsewhere[19].

The main acupuncture points were inserted bilaterally (Figure 1). ST 40, LV3 and most of the EX-LE-10 "Bafeng" points are located in the innervation area of the peroneal nerve. SP 6 and KI 3 are located close to the tibial nerve. The acupuncture treatment was carried out with sterile, single use, stainless-steel 0, 25 mm × 30 mm (manufactured by Dong Bang AcuPrime) and 0, 25 mm × 40 mm needles (manufactured by PHOENIX).

This research focuses on improvement of sensory loss assessed with patient reported outcomes, clinical and neurophysiological outcomes: Changes in the neuropathy symptom score (NSS), neuropathy deficit score (NDS), the clinical total neuropathy score (TNSc), and the patient questionnaire neuropathic pain symptom inventory (NPSI), which uses an 11-point scale to capture 5 different subdimensions of neuropathic pain which are sensitive to treatment[21]. Since the NPSI does not assess hypesthesia, we added the 11-point numeric rating scale (NRS) for patients to rate the numbness sensation on the soles of the feet. All outcomes were assessed at 8, 16 and 24 wk.

Neurophysiological assessment included measurements of the sural nerve. This nerve is typically affected in DPN and is commonly used in the diagnosis or to assess the progression of the disease[22-24]. Amplitude of the sensory nerve action potential (SNAP) and the NCV of the suralis nerve were measured with the handheld point-of-care device (POCD) NC-stat®/DPNCheck™ (NeuroMetrix, Inc., Waltham, MA). This is a novel handheld point-of-care-device which automatically performs multiple single measurements with increasing intensity within 10 s to provide amplitude (μV) and velocity (m/s) of the sural nerve after supramaximal stimulation. The POCD utilizes a linear temperature compensation method for velocity. With the default skin temperature set to 28 °C, the device automatically adjusts the velocity by 1.0 m/s per degree, with a maximum correction of 5 m/s. If skin temperature is lower than 23 °C or wrong placement or limb are recognized, the POCD displays a warning.

The device has been validated in multiple studies as an accurate screening and monitoring tool for DPN and diabetic foot risk assessment[22,25-27]. Six trained researchers (four medical doctors and two medical students) performed the measurements in a standardized manner.

To collect the data, patients were lying on their side, with the leg to be examined on top and exposing the ankle. The POCD was manually programmed to assess the left or right leg of the patient. Electrode gel was applied to the stimulating probe before placing it behind the malleolus externus. The release button was pushed, and the device would automatically give up to 16 electric discharges of increasing intensity for supramaximal stimulation. The displayed values were noted in the case report file. Each leg was measured a minimum of two times, and if values were very discrepant a third or fourth measurement was added. The procedure was then repeated for the other leg.

The primary outcome of the ACUDPN trial was a VAS for overall DPN-related complaints including pain at week 8. The results of further secondary outcomes related to pain and quality of life were published elsewhere[19].

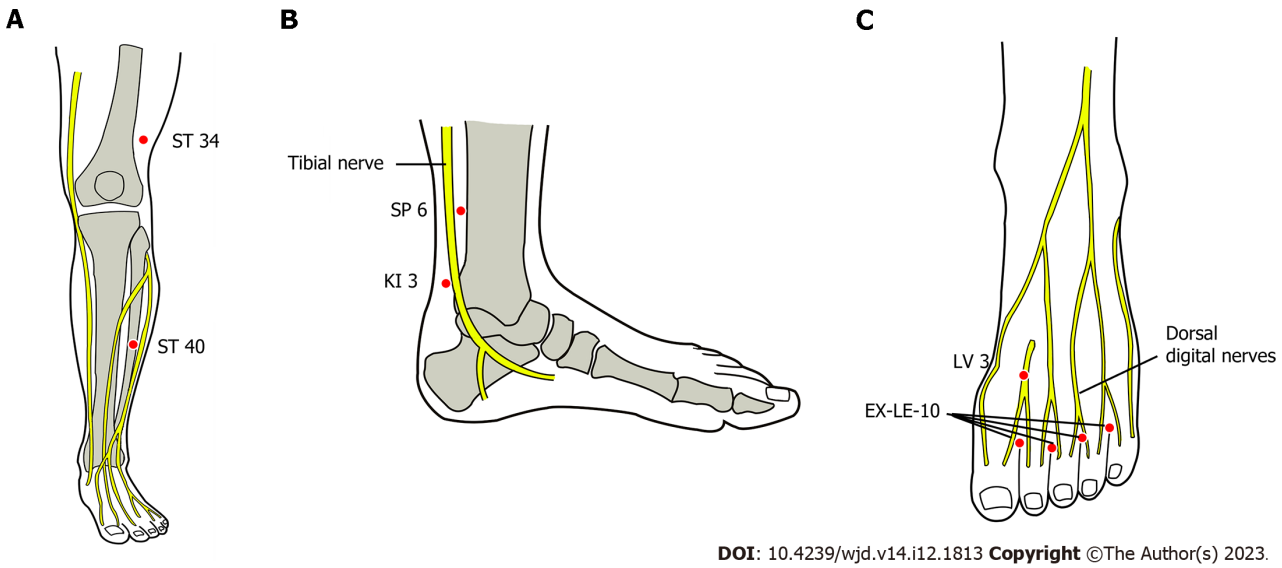

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Figure 1 Mandatory acupuncture points used in the acupuncture in diabetic peripheral neuropathy study. Anatomical presentation of acupuncture points in relation to major nerves of the lower extremity; A: Anterior aspect of leg with points on stomach-meridian 34 and 40; B: Medial aspect of ankle with point 6 on spleen-meridian and point 3 on kidney–meridian; C: Dorsal foot with point 3 on liver-merdian and extra-points lower-extremity. ST: Stomach-meridian; SP: Spleen-meridian; KI: Kidney–meridian; LV: Liver-merdian; EX-LE: Extra-points lower-extremity.

Statistical analysis

Sample size was calculated to detect a group difference in the mean values of the primary outcome parameter VAS DPN-related overall complaints after 8 wk[19,20]. A sample size of 90 patients was calculated to provide 80% power plus 15% to account for estimated dropouts (45 per group).

Evaluation of the secondary outcome parameters was exploratory, therefore was carried out without adjustment for multiple testing. All endpoints were analyzed with an ANCOVA or logistic regression (depending on the scale of the outcome). The treatment group and study center were used as fixed-effect factors and the corresponding baseline values were defined as fixed covariates. The analysis of the primary endpoint was performed with the full analysis set based on an intention-to-treat principle, the results have been published elsewhere[19]. Missing values were not replaced.

Additionally, the percentage change for the NRS 11 numbness was calculated for weeks 8 and 16 based on NRS 11 results obtained from the adjusted ANCOVA models. Data assessment was performed using SAS for Windows, version 9.4 or higher (SAS Institute, Cary, NC, United States), SPSS version 26 or higher (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp). The statistician was blinded to the groups until the end of analysis.

The trial was terminated prematurely due to strong restrictions on research with direct patient contact caused by the coronavirus disease 2019 (COVID-19) pandemic. Consequently, the previously calculated sample was not reached. Due to the lower sample size, the study center was not included as fixed effect in the statistical models for primary and secondary endpoints in the predefined statistical analysis plan. Instead, study center was included as a random effect in the analyses. The inclusion of a patient with an hemoglobin A1c below 6.5% represented a further protocol deviation with no impact on statistical analysis.

RESULTS

Altogether, 292 patients were screened for eligibility, 230 were excluded mainly because the required pathological NCV was not met or nerve conduction was within the normal age range; one patient was excluded because of very severe neuropathy with atrophy of the proximal leg muscles. Sixty-two participants [mean age 68.1 (SD 7.4); males 49 (79.0%), females 13 (21.0%)] met our inclusion criteria and were randomized 1:1 to either the intervention group or the waiting group. Recruitment was from February 2019 through to November 2020. Regarding the dropouts, 2 patients discontinued the trial in the control group because of the COVID-19 pandemic, and 3 dropped out of the intervention group, one due to intolerance to the acupuncture and 2 because of the pandemic (Figure 2, Table 1). Regarding the baseline parameters, there were no differences in demographic parameters. The control group had a few more participants with painful and severe neuropathy. Even though the relative difference could be considered significant, the absolute difference in groups was too small to address them through sensitivity analysis. Baseline characteristics are summarized in Table 1. The adherence rate was high; 96,8% of all 744 acupuncture treatments sessions were carried out.

Numbness 11-point NRS

The evaluation yielded a difference of 2.3 ($P < 0.001$) in favor of the acupuncture group, an effect that persisted until week 16 with a difference of 2.2 ($P < 0.001$), which corresponds to 35.4% and 32.4% improvement for the intervention group

Table 1 Baseline characteristics, *n* (%)

Characteristics	Acupuncture group (*n* = 31)	Control group (*n* = 31)
Age mean (SD)	66.7 (7.6)	69.5 (7.2)
Age group > 60 yr	24 (77.4)	27 (87.1)
Male	25 (80.6)	24 (77.4)
BMI > 25 kg/m²	27 (87.1)	27 (87.1)
Duration of neuropathy symptoms > 5 yr	14 (45.2)	19 (61.3)
Types of neuropathy		
Painful neuropathy	4 (12.9)	7 (22.6)
Mixed neuropathy	27 (87.1)	24 (77.4)
Severe neuropathy	3 (10.0)	7 (24.1)
Palpable pedal pulse right	29 (93.6)	25 (80.7)
Palpable pedal pulse left	25 (83.3)	26 (86.7)
Relevant comorbidities (cardiovascular, hyperlipidemia, arthrosis)	27 (87.1)	27 (87.1)
Previous treatment with medication	12 (38.1)	13 (41.9)
NPSI total intensity score		
Mean (SD)	28.1 ± 15.8	28.8 ± 20.5
Median (range)	30 (4-63)	23 (0-72)
11-NRS scale for numbness		
Mean (SD)	5.8 ± 1.8	5.8 ± 2.0
Median (range)	6 (0-9)	6 (1-8)
NDS		
Mean (SD)	8.1 ± 2.6	8.1 ± 1.9
Median (range)	9 (2-10)	8 (4-10)
NSS		
Mean (SD)	7.2 ± 1.8	7.6 ± 1.5
Median (range)	7 (4-10)	7 (5-10)
TNSc Total		
Mean (SD)	10.2 ± 4.1	11.3 ± 3.9
Median (range)	11 (2-17)	11 (6-19)
NSS Sub-groups		
Mild	3 (9.7)	0 (0.0)
Moderate	8 (25.8)	6 (19.3)
Severe	20 (64.5)	25 (80.7)
Sural nerve conduct velocity of both legs (m/s)		
Mean (SD)	40.9 ± 13.6	33.6 ± 19.9
Median (range)	44 (0-59)	44 (0-55)
Sural nerve Amplitude of the sensory nerve action potential of both legs (μV)		
Mean (SD)	4.0 ± 1.9	3.7 ± 2.1
Median (range)	4 (0-7)	4 (0-7)

TENS: Transcutaneous electrical nerve stimulation; NPSI: Neuropathy symptom pain inventory; NRS: Numeric rating scale; NDS: Neuropathy deficit score; NSS: Neuropathy symptom score; TNSc: Total clinical ceuropathy score; BMI: Body mass index.

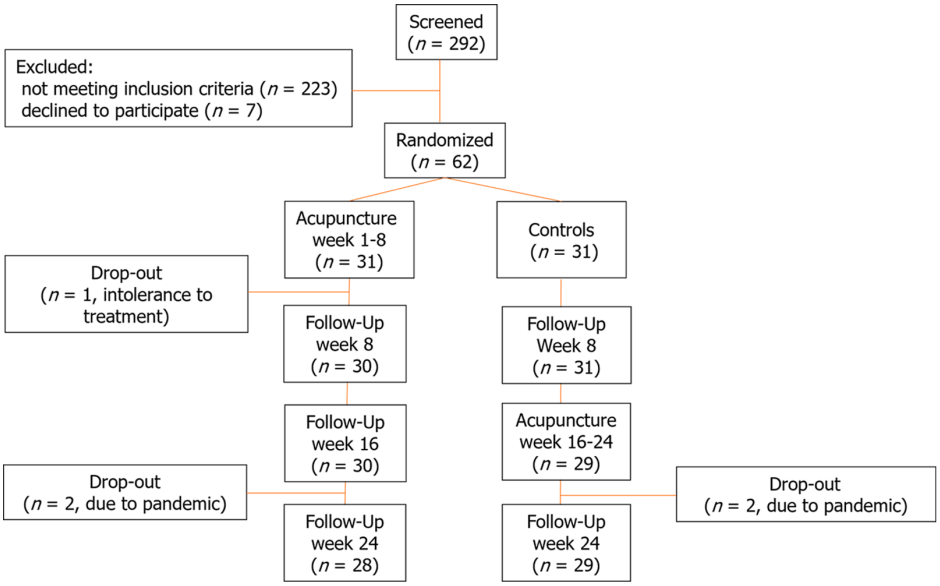

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Figure 2 Flow-chart of the acupuncture in diabetic peripheral neuropathy-trial.

compared to control at weeks 8 and 16 respectively (Figure 3). At week 24, in the acupuncture group the reduction of subjectively perceived hypesthesia was still 1.8 points lower than at baseline (Table 2).

The neuropathy pain symptom inventory

The score was improved in the acupuncture group by 12.6 points ($P < 0.001$) at week 8 and the difference persisted with 11.7 points at week 16 compared to the control group. The most notable difference was in the subdimension of paresthesia and dysesthesia at week 8 and in the subdimension of paroxysmal pain in week 16. The results of the NPSI have already been published together with the primary endpoint and the other patient reported outcomes, but the results are provided again together with the NRS-11 on numbness to complete the report of subjectively perceived changes of the neuropathy[27].

The neurological examination total neuropathy score clinical TNSc

The score improved for the acupuncture group at week 8, with a difference of 2.0 points ($P < 0.001$) compared to the control group. Effects were persistent in week 16 with a difference of 1.8 points. Pre-post-comparisons in the acupuncture group showed persisting reductions of TNSc in week 24 in the acupuncture group compared to baseline 10.2 ± 4.1 *vs*. 7.9 ± 3 .5 (Table 2).

The NSS

The NSS total score at week 8 was in favor of the acupuncture group with a difference of 1.3 ($P < 0.001$); this effect was persistent at week 16 with a difference of 1.4 ($P = 0.005$). The NDS in the acupuncture group showed a better outcome in week 8 with a difference of 1.0 points. This effect was persistent in week 16 with a difference of 0.9.

Neurophysiological data

There was no considerable difference at week 8 in the mean nervus suralis conduction velocity between the acupuncture group and the control group with 0.4 m/s ($P = 0.818$). The amplitude of the nervus suralis at week 8 showed no difference between the groups with 0.6 μV ($P = 0.156$). With the mean velocities at 38.8 m/s for both groups, the lack of effect persisted in week 16. Similarly, there was no difference in amplitude between groups at week 16 [difference of 0.4 μV ($P = 0.428$)]. At week 24 the intervention group has a mean velocity of 41.1 ± 11.8 and an amplitude of 4.6 ± 2.0.

At the end of the study intervention at week 8, the difference between groups regarding the primary outcome VAS overall DPN-related complaints was 24.7 mm (95%CI 14.8; 34.7, $P < 0.001$) in favor of the acupuncture group. The change in VAS overall DPN-related complaints at week 8 compared to baseline was 34.8 (95%CI 27.8; 41.8) in the acupuncture group *vs*. 59.5 (95%CI 52.4; 66.6) in the control group; significant improvements persisted in week 16 and clinically relevant improvements until week 24. Relevant reductions of neurological deficit scores persisted until week 24 (Table 3) For details regarding pain and quality of life outcomes see previous publication[19].

Adverse events (AE)

The following AEs were reported during the study: one patient discontinued acupuncture due to persistent discomfort after the needling session, which resolved in over one week and did not require any medical attention. Further AE were mild and consisted of minor local hematomas ($n = 18$), transient pain at needling site ($n = 5$) transient paresthesia ($n = 7$), tiredness after treatments ($n = 5$), light-headedness ($n = 1$), transient intensifying of DPN-related symptoms ($n = 4$),

Table 2 Overview of the outcome parameters

Outcome	Week	Acupuncture group adj. mean (95%CI)	Control group adj. mean(95%CI)	Difference adj. mean (95%CI)	*P* value
NRS 11 numbness	8	4.2 (3.1; 5.2)	6.5 (5.4; 7.6)	2.3 (1.3; 3.4)	< 0.001
	16	4.6 (3.7; 5.6)	6.8 (5.8; 7.9)	2.2 (1.3; 3.1)	< 0.001
NPSI	8	17.4 (13.5; 21.4)	30.0 (26.0; 34.0)	12.6 (7.1; 18.0)	< 0.001
	16	20.9 (16.2; 25.6)	32.6 (27.8; 37.4)	11.7 (5.0; 18.4)	< 0.001
NDS	8	7.0 (6.1; 8.0)	8.1 (7.1; 9.1)	1.0 (0.2; 1.9)	0.021
	16	7.3 (5.7; 9.0)	8.2 (6.5; 9.9)	0.9 (0.1; 1.7)	0.035
NSS	8	6.3 (5.8; 6.8)	7.6 (7.1; 8.1)	1.3 (0.6; 2.0)	< 0.001
	16	6.0 (4.1; 7.9)	7.4 (5.4; 9.3)	1.4 (0.4; 2.3)	0.005
TNSc	8	7.8 (5.7; 9.8)	9.8 (7.7; 11.9)	2.0 (0.9; 3.1)	< 0.001
	16	8.3 (6.8; 9.8)	10.1 (8.5; 11.7)	1.8 (0.5; 3.1)	0.010
N. suralis CV (m/s)	8	38.2 (36.1; 40.4)	38.6 (36.4; 40.7)	0.4 (-2.7; 3.5)	0.818
	16	38.8 (33.5; 44.1)	38.8 (33.6; 44.1)	0.0 (-6.2; 6.3)	0.988
N. suralis ampl. (μV)	8	3.8 (3.3; 4.4)	4.4 (3.8; 4.9)	0.6 (-0.2; 1.3)	0.156
	16	4.2 (3.5; 5.0)	4.6 (4.0; 5.3)	0.4 (-0.6; 1.4)	0.428

NRS-11: 11 point numeric ratings scale; NPSI: Neuropathic pain symptom inventory; NDS: Neuropathy deficit score; NSS: Neuropathy symptom score; TNSc: Total clinical ceuropathy score; CV: Conduction velocity.

Table 3 Pre and post comparison of outcomes in the acupuncture group at last follow-up in week 24. Intervention had ended in week 8

Variable unadjusted means (SD)	Pre (baseline)	Post (week 24)
NRS_11	5.8 ± 1.8	4.5 ± 2.3
NPSI	28.1 ± 15.8	22.6 ± 16.8
NDS	8.1 ± 2.0	7.2 ± 2.9
NSS	7.2 ± 1.8	6.2 ± 2.4
TNSc	10.2 ± 4.1	7.9 ± 3.5
Nervus suralis CV (ms)	40.9 ± 13.6	41.1 ± 11.8
Nervus suralis amplitude (μV)	4.0 ± 1.9	4.6 ± 2.0

NRS-11: 11 point numeric ratings scale; NPSI: Neuropathic pain symptom inventory; NDS: Neuropathy deficit score; NSS: Neuropathy symptom score; TNSc: Total clinical ceuropathy score; CV: Conduction velocity.

cramps of thigh muscle after needling (n = 1) and itching at needling site (n = 1). Serious AE were not observed.

DISCUSSION

Summary of results

The results of this trial suggest that patients with moderate to severe DPN-related complaints benefit from a series of acupuncture treatments with reduction of dysesthesia and improvement of sensory function; the patient-reported outcomes are reflected in the neurological examinations. The improvements persist for up to 4 mo. Changes of nerve conduction parameters could not be shown with the POCD used in this trial.

Strength and limitations

This trial focuses on hypesthesia/numbness and loss of further sensory functions, which is crucial in the development of gait disorders, risk of falls and development of diabetic foot. This approach is a novelty in DPN research, which usually concentrates on reducing pain or tingling. The reduction of numbness is of equally high importance since there is no

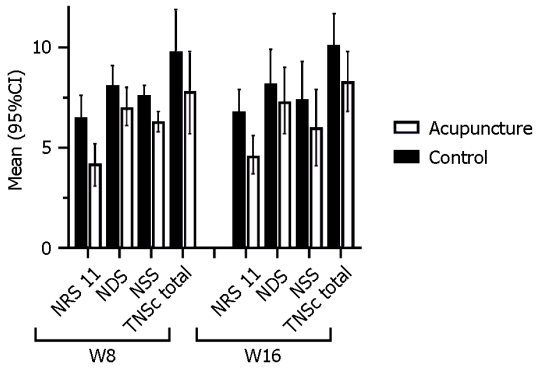

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Figure 3 11 point numeric ratings scale, neuropathic pain symptom inventory neuropathy deficit score, neuropathy symptom score, total neuropathy score clinical, week 8 and week 16. NRS-11: 11 point numeric ratings scale; NDS: Neuropathy deficit score; NSS: Neuropathy symptom score; TNSc: Total neuropathy score clinical.

pharmacological option for this symptom and it is of high relevance in protecting the integrity of the feet. Our results showed the decrease of numbness at 8 and 16 wk to be above 32%, which is indicative of a clinically important improvement. Regarding the neurological examination scores, they have been assessed by different members of the two study centers, and the findings were consistent. The trial adds the important information that a lasting improvement of DPN-related symptoms and improvement of sensory function can be achieved in chronically ill patients with an ongoing risk factor for neuropathy. The high adherence rate shows again that repetitive acupuncture treatments were well tolerated and led to patient satisfaction.

However, this study has some limitations: The sample was smaller than initially planned; a bigger trial would be useful. The statistical analysis was adapted accordingly. The results are from an analysis of secondary endpoints, which is exploratory by nature. Furthermore, a placebo effect on the patient-related outcome must be considered, despite the persistent improvement 4 mo after the end of the treatment. Due to limited resources, the clinical assessments could not be performed through a blinded assessor. Furthermore, the trial failed to show these improvements in the neurophysiological endpoints, which might be due to the diagnostic device used, which is a POCD instead of conventional NCS. Although the DPN-check device has been validated as an accurate screening tool in previous studies, it´s reliability to monitor therapy response in an intervention study has not been evaluated.

Comparison with other studies

The ACUDIN trial was a three-armed randomized, placebo-controlled trial with 180 patients with confirmed type 2 diabetes-induced DPN that were either allocated to receive 10 sessions of needle acupuncture, laser acupuncture, or placebo laser acupuncture for 10 consecutive weeks[17]. Neurological assessments, including NCS of sural and tibial nerves with the conventional method, were performed at baseline and weeks 6 and 15 with conventional neurography. Primary outcome was the delta of sural SNAP. Secondary outcomes included further NCS values, clinical scores, and patient-reported outcome measures (PROMs). The amplitude of the sural nerve improved by 1.95 in the acupuncture group *vs* 0.5 in the placebo group and the sural nerve conduction velocities improved significantly by a mean of 13.5 m/s in the acupuncture group compared to placebo lase with 3.4 m/s. However, sural nerve conduction and amplitude was evaluated in the ACUDIN with classical needle neurography by experienced neurologists, which might be more sensitive.

A systematic review with meta-analysis of 14 randomized control trials and 1 long-term follow-up study on the use of acupuncture for various neuropathies showed that acupuncture was effective for diabetic neuropathy, Bell's palsy, and carpal tunnel syndrome[28]. In the 4 Chinese trials on diabetic neuropathy neurological examination scores and conventional nerve conduction study improved significantly. In a study with a design comparable to ACUDPN with a randomized waiting-list control group, 87 Chinese patients with chemotherapy-induced neuropathy were investigated. Within this cohort, 10 acupuncture sessions resulted in significant clinical improvements after 8 wk regarding the primary outcome (pain) and also neurological assessment[29]. A subset of patients was tested with NCS and had no or only borderline electrophysiological signs of neuropathy at baseline. These parameters did not change after 8 wk, the authors linked it to the small number of patients in this subset and to the absence of neurophysiological abnormal findings at baseline. However, the randomized crossover ACUCIN study with 60 patients with chemotherapy-induced neuropathy revealed improvement in NCS measured by classical neurography and PROMs[30].

Further research

The results of this trial showed beneficial effects in type 2 DPN, however there were several limitations which must be addressed in future research. Future trials should be conducted with a sham-control group and proper blinding, and with neurological outcomes as primary endpoint and conventional NCS in the secondary outcomes. Studies with larger sample size would be needed to address the potential impact on the study result by neuropathy subtype.

CONCLUSION

Results of this exploratory study suggest that body acupuncture seems to have a positive effect on DPN-related neurological impairment. In this study, acupuncture was generally safe. Improvement in numbness and clinical examination scores persist until 2 mo after end of treatment in a clinically significant way in comparison to the control group. High-quality randomized and sham-controlled clinical trials with adequate patient number are much needed to confirm these results and to identify specific effects.

ARTICLE HIGHLIGHTS

Research background

Peripheral neuropathy in patients with diabetes type 2 is common. It can lead to loss of sensory function which increases the risk of gait disturbances and falls, or injuries of the feet that go unnoticed. Painful sensations occur frequently in diabetic peripheral neuropathy (DPN) which have an impact on quality of life.

Research motivation

Non-pharmacological therapeutic options are warranted for a patient group who is already confronted with polypharmacy. Pain control as well as restoration of sensory function are the motivation to evaluate the effects of acupuncture treatments on outcomes of neuropathy of the lower extremities.

Research objectives

What are the effects of 12 acupuncture treatments administered over the course of 8 wk on the subdimension of neuropathy, such as pain, tingling and numbness? Are the treatments safe and well-tolerated? How long do the effects last over the course of 24 wk?

Research methods

Open, multicenter, randomized controlled trial with patients suffering from diabetes type 2 and moderate to severe peripheral neuropathy.

Research results

Sixty-two participants were included and randomized in the two trial arms. A numeric rating scale for numbness, neuropathy symptom scores and clinical examinations showed significant and relevant improvements of neuropathy at week 8. Improvements remained significant up to 16 wk after the end of the intervention. No changes could be recorded in the nerve conduction studies. Only minor adverse events were recorded.

Research conclusions

Body acupuncture seems to have a positive effect on DPN-related neurological impairment. Improvement in numbness and clinical examination scores persist until 2 mo after end of treatment in a clinically significant way in comparison to the control group.

Research perspectives

Sham-controlled clinical trials with adequate patient numbers are needed to confirm these results and to identify specific effects.

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FOOTNOTES

Author contributions: The study was conceptualized and coordinated by Dietzel J, Brinkhaus B, Hahn K, Willich SN and Ortiz M; Dietzel J, Habermann IV, Hoerder S, Ortiz M, Meyer-Hamme G, and Schroeder S acquired the patient data and performed the treatments; Dietzel J, Roll S and Grabowska W conducted the main data analysis; Brinkhaus B had the overall medical responsibility, and Brinkhaus B and Schroeder S were equally supervising the trial; Hoerder S and Dietzel J prepared the manuscript.

Institutional review board statement: The study protocol was approved by the ethics committee Berlin (EA1/183/18), Germany in October 2018. ACUDPN was performed in compliance with the Declaration of Helsinki and standards of Good Clinical Practice.

Clinical trial registration statement: The trial was registered on ClinicalTrials.gov NCT03755960.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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ORIGINAL ARTICLE

Basic Study

Depletion of gut microbiota facilitates fibroblast growth factor 21-mediated protection against acute pancreatitis in diabetic mice

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Abstract

BACKGROUND

Fibroblast growth factor 21 (*FGF21*), primarily secreted by the pancreas, liver, and adipose tissues, plays a pivotal role in regulating glucose and lipid metabolism. Acute pancreatitis (AP) is a common inflammatory disease with specific clinical manifestations. Many patients with diabetes present with concurrent inflammatory symptoms. Diabetes exacerbates intestinal permeability and intestinal inflammation, thus leading to the progression to AP. Our previous study indicated that FGF21 significantly attenuated susceptibility to AP in mice.

AIM

To investigate the potential protective role of *FGF21* against AP in diabetic mice.

METHODS

In the present study, a mouse model of AP was established in diabetic (db)/db diabetic mice through ceruletide injections. Thereafter, the protective effects of recombinant *FGF21* protein against AP were evaluated, with an emphasis on examining serum amylase (AMS) levels and pancreatic and intestinal inflammatory cytokines [interleukin (IL)-6, tumor necrosis factor-alpha (TNF-), and intestinal IL-1β]. Additionally, the impact of this treatment on the histopathologic changes of the pancreas and small intestinal was examined to elucidate the role of *FGF21* in diabetic mice with AP. An antibiotic (Abx) cocktail was administered in combination with *FGF21* therapy to investigate whether the effect of *FGF21* on AP

in diabetic mice with AP was mediated through the modulation of the gut microbiota. Subsequently, the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), a bioinformatics software package, was used to predict different pathways between the groups and to explore the potential mechanisms by which the gut microbiota influenced the protective effect of *FGF21*.

RESULTS

The results indicated that *FGF21* notably diminished the levels of serum AMS (944.5 ± 15.9 *vs* 1732 ± 83.9, $P < 0.01$) and inflammatory factors including IL-6 (0.2400 ± 0.55 *vs* 1.233 ± 0.053, $P < 0.01$), TNF- (0.7067 ± 0.22 *vs* 1.433 ± 0.051, $P < 0.01$), and IL-1β (1.377 ± 0.069 *vs* 0.3328 ± 0.02542, $P < 0.01$) in diabetic mice with AP. Moreover, notable signs of recovery were observed in the pancreatic structure of the mice. The histologic evidence of inflammation in the small intestine, including edema and villous damage, was significantly alleviated. *FGF21* also significantly altered the composition of the gut microbiota, reestablishing the *Bacteroidetes/Firmicutes* ratio. Upon treatment with an Abx cocktail to deplete the gut microbiota, the *FGF21* + Abx group showed lower levels of serum AMS (0.9328 ± 0.075 *vs* 0.2249 ± 0.023, $P < 0.01$) and inflammatory factors (1.083 ± 0.12 *vs* 0.2799 ± 0.032, $p < 0.01$) than the *FGF21* group. Furthermore, the *FGF21* + Abx group exhibited diminished injury to the pancreatic and small intestinal tissues, accompanied by a significant decrease in blood glucose levels (17.50 ± 1.1 *vs* 9.817 ± 0.69 mmol/L, $P < 0.001$). These findings underscored the superior protective effects of the combination therapy involving an Abx cocktail with *FGF21* over the *FGF21* treatment alone in diabetic mice with AP. The gut microbiota composition across different groups was further characterized, and a differential expression analysis of gene functions was undertaken using the PICRUSt2 prediction method. These findings suggested that *FGF21* could potentially confer therapeutic effects on diabetic mice with AP by modulating the sulfate reduction I pathway and the superpathway of n-acetylceramide degradation in the gut microbiota.

CONCLUSION

This study reveals the potential of *FGF21* in improving pancreatic and intestinal damage recovery, reducing blood glucose levels, and reshaping gut microbiota composition in diabetic mice with AP. Notably, the protective effects of *FGF21* are augmented when combined with the Abx cocktail.

Key Words: Acute pancreatitis; Fibroblast growth factor 21; Gut microbiota; Diabetes; PICRUSt2; Cocktail of antibiotics

Core Tip: This study reveals the potential of facilitates fibroblast growth factor 21 (*FGF21*) in improving pancreatic and intestinal damage recovery, reducing blood glucose levels, and reshaping gut microbiota composition in diabetic mice with acute pancreatitis (AP). Notably, the protective effects of *FGF21* are augmented when combined with the Abx cocktail. These findings provide new insights into the prevention and treatment of diabetes complicated by AP.

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INTRODUCTION

Acute pancreatitis (AP) is a local inflammatory disorder of the pancreas caused by aberrant activation of pancreatic proteases due to various contributing factors. The global annual incidence of AP is estimated to be approximately 34 cases per 100000 individuals, leading to many hospitalizations, high medical costs, and long-term sequelae for patients worldwide[1,2]. Diabetes is a chronic metabolic disorder caused by insufficient secretion or impaired action of insulin, leading to elevated blood glucose levels. Inflammation-related symptoms are commonly observed in many diabetic patients. Chronic inflammation is a complication of diabetes and other diseases, contributes to the occurrence and progression of diabetes and the associated conditions. The occurrence of diabetes has also been indicated to exacerbate the development of AP. Recent evidence further suggests that obesity aggravates the severity of AP, increases intestinal permeability, and facilitates intestinal inflammation[3]. Additionally, the analysis of the fecal microbiota composition revealed a reduction in the abundance of bacteria in obese rats with AP compared with rats with a normal body weight[4,5].

The imbalance in the gut microbiota composition and the reduction in microbial diversity in the intestine may lead to an increase in the pathogenic bacterial count and the disruption of cellular integrity. These alterations can contribute to an increase in intestinal leakage and permeability, leading to the subsequent development of intestinal inflammation and a reduction or disturbance in the immune response of the intestinal mucosa[6]. Prior research has highlighted that rats with

type 2 diabetes and AP undergo changes in the structure of their gut microbiota, which increases the susceptibility to complex AP injury. It is interesting to note that fecal microbiota transplantation effectively mitigates intestinal mucosal injury and reduces inflammatory cell infiltration in mice[7]. Another study has proposed the prognosis of AP could be moderately facilitated through probiotic therapy[8]. Probiotic strains can enhance the production of interleukin (IL)-10, a pivotal regulatory and anti-inflammatory cytokine in diabetic mice. IL-10 suppresses pro-inflammatory cytokines, such as interferon-gamma and IL-2/IL-1β, thereby impeding the development of low-grade inflammation and diabetes[9,10].

Fibroblast growth factor 21 (*FGF21*), a recently identified metabolic regulator secreted by the liver, adipose tissue, and pancreas, has shown potent anti-inflammatory effects in animal experiments. It can downregulate the expression of inflammatory cytokines, including tumor necrosis factor-alpha (TNF-) and IL-6[11]. Our preliminary research underscored a significant upregulation of *FGF21* expression in the context of AP. Exogenous administration of *FGF21* has been indicated to curtails pancreatic injury, aberrant expression of digestive enzymes, and inflammatory response, thus impeding the occurrence of AP[12]. However, it is important to further explore the potential of *FGF21* to ameliorate local or systemic inflammation and diminish blood glucose levels in mice with diabetes complicated by AP. Additionally, the involvement of the gut microbiota in the protective effects of *FGF21* in diabetic mice with AP warrants further investigation.

In the present study, a mouse model of AP was induced in diabetic (db)/db diabetic mice using ceruletide injections. The subsequent investigation focused on evaluating the protective effects of recombinant *FGF21* protein on serum amylase (AMS) and pancreatic and intestinal inflammatory cytokines (IL-6, TNF-, and intestinal IL-1β). Additionally, we assessed the impact of this treatment on histopathologic changes in the pancreas and small intestine, aiming to enhance understanding of the role of *FGF21* in diabetic mice with AP. The study proceeded by administering a combination of *FGF21* therapy and an antibiotic (Abx) cocktail to assess the involvement of gut microbiota in the potential impact of *FGF21* on AP in diabetic mice. Subsequently, the application of Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), a bioinformatics software package, enabled us to predict different pathways between the groups. The objective was to explore the potential mechanisms by which the gut microbiota influenced the protective effect of *FGF21*.

MATERIALS AND METHODS

Induction of AP in diabetic mice

Male diabetic mice (db/db), aged 10 wk and weighing 40-55 g, were purchased from GemPharmatech Co., Ltd. (Nanjing, Jiangsu, China) and housed at the Experimental Animal Center of Wenzhou Medical University (Zhejiang, China). All experimental protocols involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Committee on Animal Health and Care of Wenzhou Medical University. Before the experiment, all animals were provided with a normal diet and allowed to acclimatize for 1 wk under a 12:12 Light-dark cycle at room temperature (23 ± 1°C) and approximately 60% humidity. Before AP modeling, the animals were subjected to a 12-h fasting period and had *ad libitum* access to drinking water.

Mice with fasting blood glucose levels > 16.7 mmol/L were regarded as diabetic mice and were randomly divided into the following groups (n = 5 per group): Diabetic mouse group (db), ceruletide-induced AP model group (AP), *FGF21* treatment group (*FGF21*), and *FGF21* combined with Abx cocktail treatment group (*FGF21* + Abx). AP model was established in mice of the AP, *FGF21*, and *FGF21* + Abx groups, wherein each mouse received seven intraperitoneal injections of ceruletide (50 μg/kg), at hourly intervals[13,14]. The mice in the db group received intraperitoneal injections of the same volume of normal saline as a control. The successful establishment of the AP mouse model was confirmed based on the following criteria: (1) Increased activity of serum AMS released from the pancreas, wherein the enzyme was detected using an enzyme-linked immunosorbent assay kit after AP induction; (2) no increase in pancreatic AMS levels; and (3) pancreatic tissues not meeting the diagnostic criteria for pancreatitis according to the modified Schmidt scoring system.

The animals were euthanized 6 h after the final injection of ceruletide. Serum and pancreatic and intestinal tissues were collected from mice of each group to determine the serum AMS levels and the ratio of pancreas weight to body weight. The collected tissues were embedded in paraffin, sliced, and stained with hematoxylin and eosin (HE), followed by a microscopic examination to observe the morphological changes in pancreatic tissues. 16S rRNA sequencing was performed to observe changes in the gut microbiota.

FGF21 treatment

Experimental mice in the *FGF21* and *FGF21* + Abx groups received an intraperitoneal injection of *FGF21* (1 mg/kg) 1 h before the ceruletide injection. In the same manner, mice from the db and AP groups received intraperitoneal injections of normal saline as a control.

Abx cocktail treatment for diabetic mice with AP

Mice in the *FGF21* + Abx group were orally administered with an Abx cocktail of non-absorbable Abx (ampicillin, neomycin, metronidazole, and vancomycin). The Abx cocktail was prepared at concentrations of 1 g/L, 1 g/L, 1 g/L, and 0.5 mg/L for each of these Abx, respectively. The Abx cocktail solution was freshly prepared every 2 d and administered continuously for 3 wk.

After 3 wk of Abx cocktail treatment, fecal samples were collected from mice in the *FGF21* + Abx group for fecal DNA extraction[13], followed by the detection of bacteria in the intestines using universal primers[14]. After depletion of the majority of bacteria in the mouse intestine, the mice in the *FGF21* + Abx group received an intraperitoneal injection of *FGF21*, followed by an intraperitoneal injection of ceruletide 1 h later to establish the AP model in diabetic mice.

Immunoblotting assay

Total protein was extracted from the pancreatic and intestinal tissues of mice, and the protein concentration was determined using a bicinchoninic acid assay. The protein samples were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membranes were then blocked with 10% skim milk–Tris-buffered saline with Tween 20 (TBST) solution at room temperature for 1.5 h, followed by an overnight incubation at 4 °C with the corresponding primary antibodies (IL-6 antibody diluted at 1:1000, IL-1β antibody diluted at 1:1000, TNF- antibody diluted at 1:2000, β-actin antibody diluted at 1:5000, and GAPDH antibody diluted at 1:5000, all purchased from Proteintech). The membranes were rinsed with TBST solution in triplicate and then incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase at room temperature for 1 h. Chemiluminescent signals were detected using the Tanon-5200 chemiluminescence imaging system. The signal from each protein band was quantified using the ImageJ software.

Histological and immunohistochemical examinations

Before histological analysis, mouse pancreatic and small intestinal tissues were fixed with 4% paraformaldehyde for more than 24 h. The fixed tissues were then embedded in paraffin, sliced at a thickness of 5 μm, and subjected to HE staining. The stained tissue sections were mounted with neutral resin and observed under a light microscope. The modified Schmidt scoring system was applied for the quantitative evaluation of pancreatic tissue damage.

16S rRNA sequencing

The diversity of the gut microbiota in clinical or laboratory animal samples was analyzed using 16S rRNA sequencing and the next-generation microbiome bioinformatics platform QIIME 2[15]. The latest version of the QIIME 2 platform, together with the DADA2 software package, was used to denoise the sequence data using approximately 100% similarity, with an operational taxonomic unit (OTU) clustering at 97% similarity[16]. Redundancy was then removed to obtain feature data (representative sequences) for comparison with the 16S database (132 version) and NT-16. This comparison aimed to identify and annotate all 16S rRNA sequences detected in the samples, including taxonomic categories of kingdom, phylum, class, order, family, genus, and species.

Statistical analysis

Statistical analysis of data was performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). Data were presented as mean ± SEM. Statistical significance was determined using Student's *t*-test (for comparisons between two experimental conditions) or analysis of variance (ANOVA) (for comparisons among three or more experimental conditions). Pearson analysis was used to determine linear correlations between variables. A *P*-value of less than 0.05 was considered to indicate statistical significance.

RESULTS

FGF21 significantly attenuates pancreatic injury and inflammation in diabetic mice with AP

To investigate the impact of *FGF21* on AP in diabetic mice, we established an AP model in diabetic mice by administering ceruletide injections. Following the ceruletide injection, the ratio of pancreas weight to body weight of diabetic mice was notably reduced by approximately 22.1% (Figure 1A, $P < 0.01$). Serum levels of AMS in diabetic mice were found to be twice as high as those in the db group (Figure 1B, $P < 0.01$). Concurrently, the concentration of the inflammatory cytokine IL-6 in diabetic mice increased to five times of that in the db group (Figure 1C and D, $P < 0.01$), while the TNF- level showed a significantly elevated to twice that of the db group (Figure 1C and E, $P < 0.01$). Following intraperitoneal injection of recombinant human *FGF21* protein, the *FGF21* group demonstrated an elevated ratio of pancreas weight to body weight, measuring at 19.3% (Figure 1A, $P < 0.05$). This result was accompanied by a reduction in the levels of serum AMS and inflammatory cytokines IL-6 and TNF- (Figure 1C). Specifically, the serum levels of AMS decreased by 40.1% (Figure 1B, $P < 0.001$), IL-6 levels decreased by 24.4% (Figure 1D, $P < 0.01$), and TNF- levels decreased to 65.1% of those in the AP group (Figure 1E, $P < 0.05$). Furthermore, diabetic mice with AP displayed pathological changes in pancreatic tissues, such as pancreatic edema, extensive intracellular vacuolation, and cellular necrosis (Figure 1F). Conversely, histological tissue sections of mice in the *FGF21* group exhibited a significant reduction in tissue damage (Figure 1F).

These findings highlight that ceruletide injections induce pancreatic injury and inflammation in diabetic mice, while *FGF21* treatment mitigates these symptoms in diabetic mice with AP.

FGF21 treatment mitigates intestinal damage and improves the composition of gut microbiota

To explore gut microbiota alterations in the context of AP, we assessed intestinal tissue damage in mice. The mice in the AP group showed exacerbated histologic evidence of inflammation in the small intestine, characterized by tissue edema, increased villus width, and villus damage (Figure 2A). After *FGF21* treatment, noticeable reductions in the levels of inflammatory factors were observed in the small intestinal tissue (Figure 2B), with TNF- levels decreasing to 38.4% of that

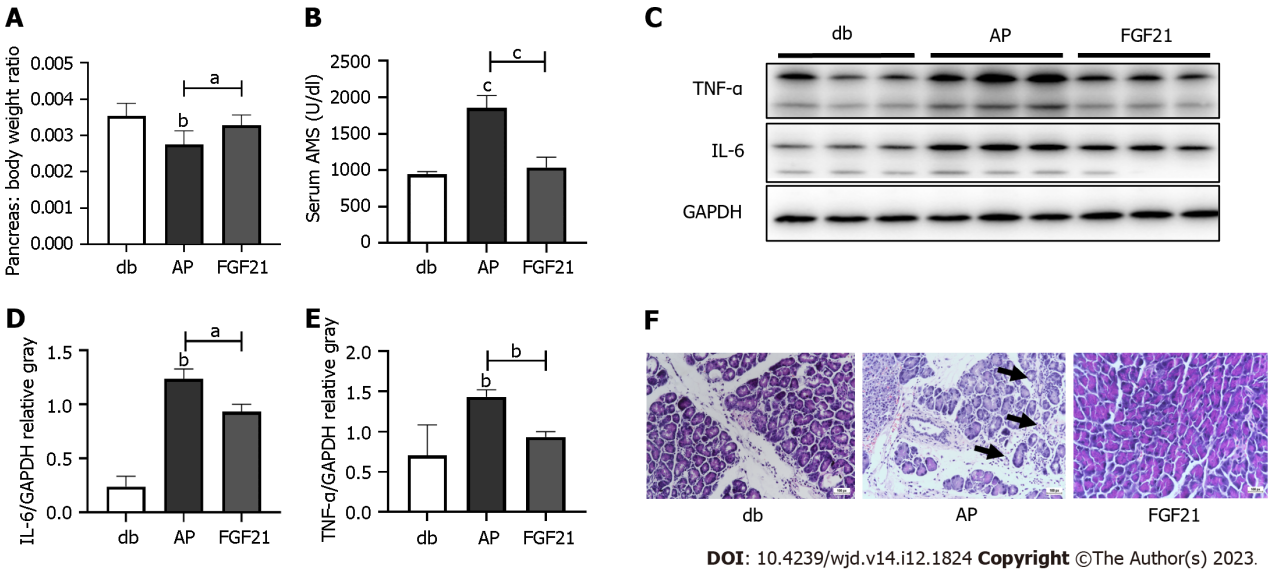

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Figure 1 Fibroblast growth factor 21 significantly attenuates pancreatic injury and inflammation in diabetic mice with acute pancreatitis. A: The ratio of pancreas weight to body weight of mice in diabetic (db), acute pancreatitis (AP) and fibroblast growth factor 21 (*FGF21*) groups; B: Serum levels of amylase of mice in db, AP and *FGF21* groups; C-E: Representative immunoblots of inflammatory factors in mouse pancreatic tissue. Expression levels of interleukin-6 and tumor necrosis factor-alpha were quantified using densitometry, with GAPDH as a protein loading control; F: Pathological changes in pancreatic tissues of mice in db, AP and *FGF21* groups, such as pancreatic edema, extensive intracellular vacuolation, and cellular necrosis (scale bar: 100 μm). Data are presented as mean ± SD, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. AP: Acute pancreatitis; AMS: Amylase; db: Diabetic; IL: Interleukin; TNF-: Tumor necrosis factor-alpha; *FGF21*: Fibroblast growth factor 21.

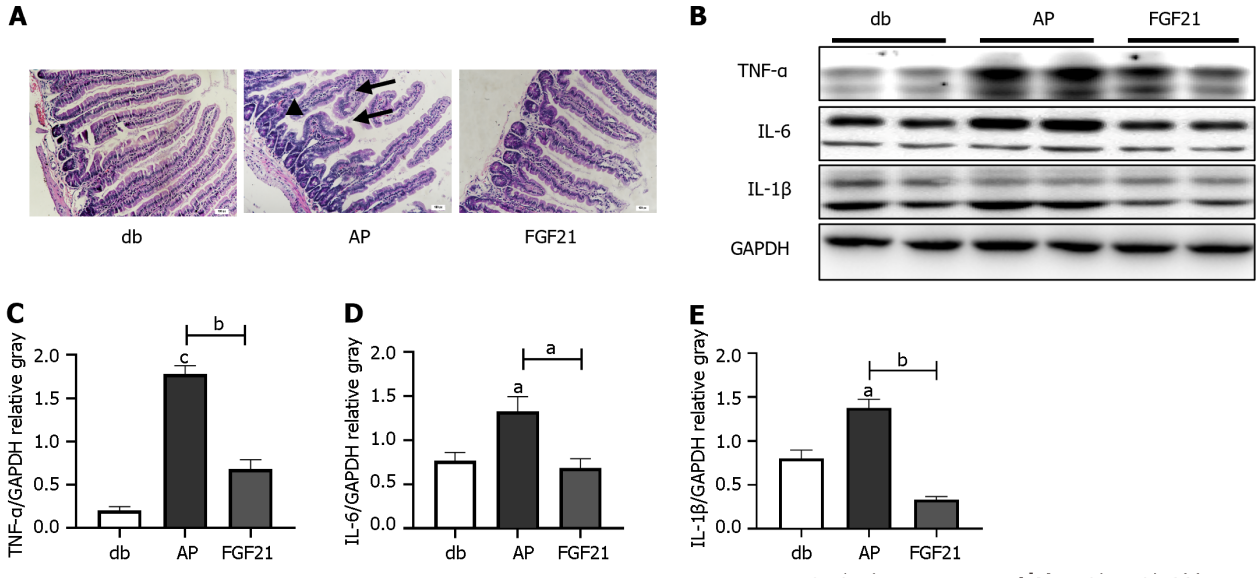

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Figure 2 Fibroblast growth factor 21 treatment mitigates intestinal damage and inflammation in diabetic mice with acute pancreatitis. A: Histological changes in the small intestine of mice in diabetic, acute pancreatitis and fibroblast growth factor 21 groups, characterized by tissue edema, increased villus width, and villus damage (scale bar: 100 μm); B-E: Representative immunoblots of inflammatory factors in mouse small intestinal tissue. Expression levels of interleukin-6 and tumor necrosis factor-alpha were quantified using densitometry, with GAPDH as a protein loading control. Data are presented as mean ± SD, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. AP: Acute pancreatitis; db: Diabetic; IL: Interleukin; TNF-: Tumor necrosis factor-alpha; *FGF21*: Fibroblast growth factor 21.

in the AP group (Figure 2C, $P < 0.01$), IL-6 levels decreasing by half (Figure 2D, $P < 0.05$), and IL-1β levels decreasing to 24.2% of that in the AP group (Figure 2E, $P < 0.01$), indicating a significant alleviation of intestinal tissue damage.

Next, 16S rRNA sequencing was performed to examine whether *FGF21* altered the composition of the gut microbiota in diabetic mice while alleviating intestinal damage. Principal coordinate analysis (PCoA) results demonstrated distinct segregation of the microbial communities among the db, AP, and *FGF21* groups, underscoring differences in the gut microbiota composition among the three groups (Figure 3A, $P < 0.01$). Alpha diversity of the gut microbiota, which serves as a comprehensive index of species abundance and evenness in community ecology, was assessed using four commonly used indices: observed OTUs, Chao1, Shannon, and Simpson indices. The observed OTUs and Chao1 indices reflect the

species abundance in a sample, while the Shannon and Simpson indices reflect both the species abundance and evenness. When compared with the db group, all four indices showed significant increases in the AP group ($P < 0.001$). The *FGF21* group exhibited notable decreases in OTUs and Chao1 indices when compared with the AP group ($P < 0.05$), with Shannon and Simpson indices showing a nonsignificant decrease (Figure 3B-E). These results indicate an increase in gut microbiota abundance in diabetic mice with AP, and *FGF21* treatment could reverse this change. The gut microbiota plays a pivotal role in the occurrence of AP in diabetic mice.

We further compared the alterations in bacterial communities among the three groups, with the stacked bar chart illustrating bacterial species distribution and changes in species composition and distribution within each group. The experimental results highlighted distinct variations in the highest relative abundance of the top 30 species at different taxonomic levels of the samples. At the phylum level, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* emerged as major taxa in abundance across all samples (Figure 3F). Notably, phylum *Firmicutes* exhibited a marked elevation in the AP group (Figure 3G, $P < 0.01$), with significant decreases in the *FGF21* group (Figure 3G, $P < 0.001$). Meanwhile, phyla *Proteobacteria* and *Bacteroidetes* showed significant decreases in the AP group (Figure 3G, $P < 0.05$) and significant increases in the *FGF21* group (Figure 3G, $P < 0.05$). Moreover, *Firmicutes* was found to be the most abundant phylum across all samples. This study found that the *Bacteroides/Firmicutes* ratio decreased in the AP group, with a rebound in the *FGF21* group.

Combined therapy of Abx cocktail and FGF21 significantly decreases the susceptibility to AP in diabetic mice

Upon disrupting the gut microbiota through an Abx cocktail, a notable decrease in bacterial abundance was observed in the mouse feces (Figure 4A). Following the commencement of Abx cocktail treatment, the body weight of mice decreased, reaching the lowest point on day 13, with an average body weight of 45 g. However, following adaptation to the Abx cocktail feeding, the body weight of mice gradually increased and reached 48 g (Figure 4B). In subsequent experiments, when compared with the AP group, the *FGF21* + Abx group demonstrated a significant decrease in serum AMS levels (Figure 4C, $P < 0.0001$), with minimal damage observed in pancreatic and intestinal tissue sections (Figure 4D and E). Immunoblotting analysis revealed further reductions in the levels of the pro-inflammatory cytokines TNF- and IL-6 in the *FGF21* + Abx group in the pancreatic tissue when compared with those in the *FGF21* group (Figure 4F). Specifically, TNF- exhibited a significant decrease of 75.9% (Figure 4G, $P < 0.01$), and IL-6 showed a reduction to 25.8% of the *FGF21* + Abx group (Figure 4H, $P < 0.01$). Similarly, noticeable reductions in the levels of inflammatory factors were observed in the small intestinal tissue (Figure 4I), with TNF- levels decreasing to 23.4% of that in the *FGF21* group (Figure 4J, $P < 0.001$), IL-1β levels decreasing by half (Figure 4K, $P < 0.05$), and IL-6 levels decreasing to 45.6% (Figure 4L, $P < 0.01$). Notably, blood glucose levels significantly decreased from 17.50 ± 1.1 to 9.817 ± 0.69 mmol/L (Figure 4F, $P < 0.001$) in the *FGF21* + Abx group, further decreasing from 15.14 ± 1.8 mmol/L in the *FGF21* group (Figure 4M, $P < 0.05$). These experimental results demonstrate that the combination therapy of Abx cocktail with *FGF21* exerts a more potent protective effect on AP in diabetic mice compared to *FGF21* treatment alone. The Abx cocktail enhances the protective efficacy of *FGF21* in diabetic mice with AP.

Abx cocktail combined with FGF21 treatment alters microbiota in diabetic mice with AP

To further investigate the contribution of the microbiota to the protective effects of *FGF21* in diabetic mice with AP, we compared the changes in bacterial communities among four groups of mice (Figure 5A and B). At the phylum level, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* were the major taxa of the bacterial communities across all four groups, accounting for 96.6% of the total abundance. In comparison with the db group, phylum *Firmicutes* showed a substantial increase to 66.2% in the AP group (Figure 5B, $P < 0.01$), while it decreased to 43.7% in the *FGF21* group, reaching normal levels, and further decreased to 5.7% in the *FGF21* + Abx group. Relative to the db group, phyla *Proteobacteria* and *Bacteroidetes* significantly decreased to 10.9% and 20.7%, respectively, in the AP group (Figure 5B, $P < 0.05$). After *FGF21* treatment, the abundance of *Proteobacteria* and *Bacteroidetes* significantly increased to 18.9% and 35.5%, respectively, in the *FGF21* group (Figure 5B, $P < 0.05$), reaching normal levels. In contrast, in the *FGF21* + Abx group, the phylum *Proteobacteria* significantly increased to 87.3% (Figure 5B, $P < 0.001$), while the phylum *Bacteroidetes* significantly decreased to 1.3% (Figure 5B, $P < 0.001$). Additionally, significant differences in gut microbiota composition were observed at the genus level among different groups (Figure 5C and D). The predominant taxa in the gut microbiota of the four groups of mice were *Lactobacillus*, *Mucispirillum-Klebsiella*, and *Escherichia coli-Shigella*, accounting for 44.3% in the *FGF21* + Abx group. Moreover, the AP group exhibited the highest abundance of *Lactobacillus*, accounting for 53%. In the *FGF21* + Abx group, *Escherichia coli-Shigella* accounted for 42%, while its abundance remained below 1% in the other three groups. Notably, prior research has highlighted an increase in the abundance of the phylum *Firmicutes* (gram-positive bacteria) and a decrease in the abundance of the phylum *Bacteroidetes* in obese mice[13]. After *FGF21* treatment, the proportion of *Firmicutes* was significantly reduced, while that of *Bacteroidetes* was significantly increased in diabetic mice with AP, underscoring the effectiveness of *FGF21* in alleviating diabetes conditions in diabetic mice with AP.

Through linear discriminant analysis (LDA) effect size (LEfSe), the biomarkers were compared among the four groups of samples. In this study, a threshold of > 4.5 LDA score and $P < 0.05$ were set for the LEfSe analysis. The variance in LDA scores across the four groups of microbiota samples revealed that the phylum *Proteobacteria*, order Enterobacteriales, and family Enterobacteriaceae were the most abundant species in the *FGF21* + Abx group. *Escherichia coli-Shigella* was found to be the most abundant species in the *FGF21* group. The phylum *Firmicutes*, class Clostridia, and order Clostridiales were most abundant in the db group. These bacteria may serve as potential targets for the treatment of diabetes complicated with AP. The order Lactobacillales, family Lactobacillaceae, and genus *Lactobacillus* were the most abundant in the AP group (Figure 5E). These bacteria belong to the class Bacilli. The phylogenetic tree in Figure 5F shows the origins of the microbiota at different taxonomic levels.

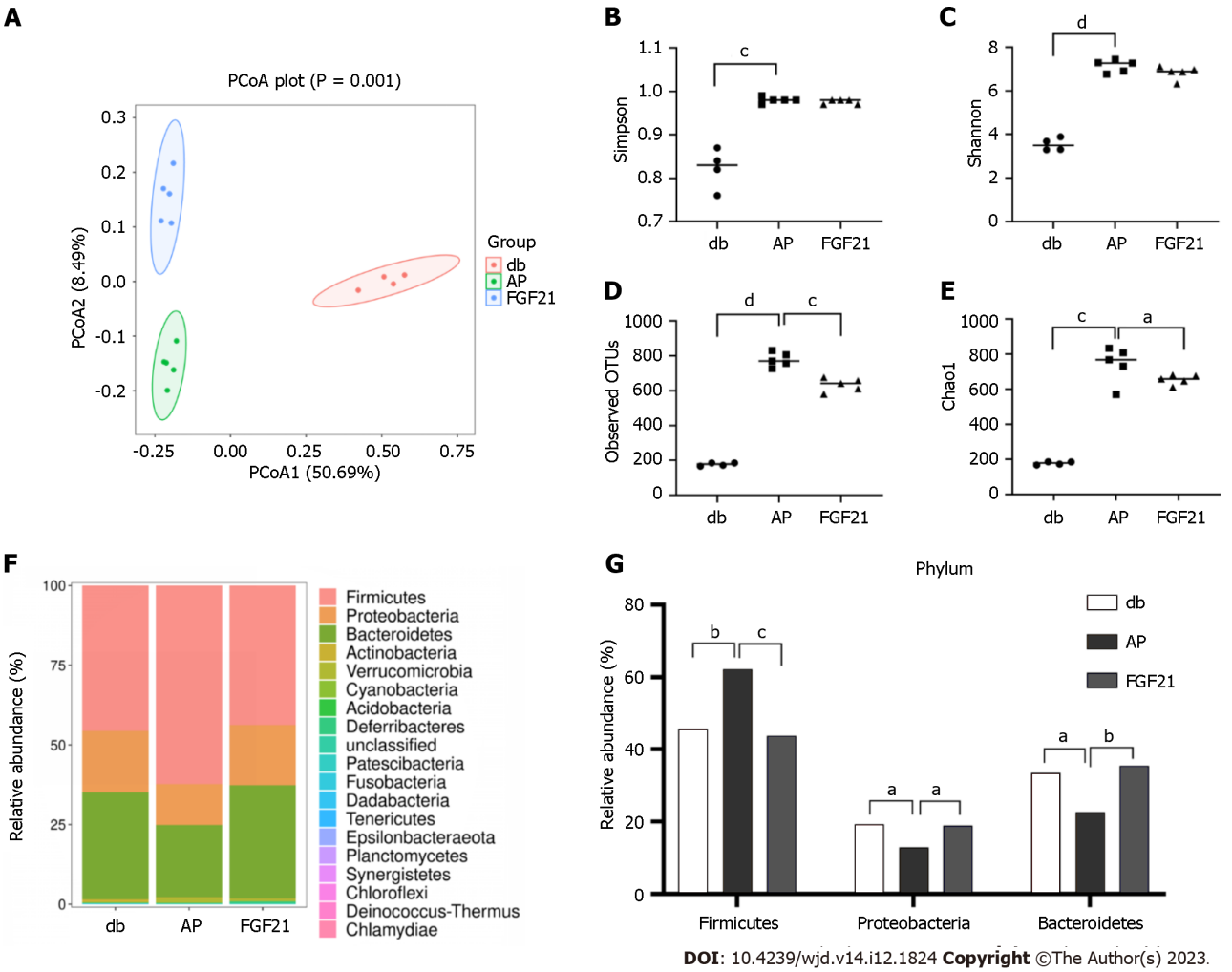

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Figure 3 Fibroblast growth factor 21 treatment improves the composition of gut microbiota. A: Principal coordinate analysis results demonstrated distinct segregation of the microbial communities among the diabetic (db), acute pancreatitis (AP), and fibroblast growth factor 21 (*FGF21*) groups. Different colors in the scatter plots represent samples from different groups; the higher the similarity between samples, the closer they are in the plots; B-E: The observed operational taxonomic units, Chao1, Shannon, and Simpson indices of the gut microbiota of db, AP and *FGF21* group mice. All four indices increased in the AP group compared with the db group, and the *FGF21* group exhibited decreases compared with the AP group; F: Bar graph of the structural distributions of fecal microbial communities at the phylum level; G: Relative abundance of the dominant phyla. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$. AP: Acute pancreatitis; db: Diabetic; IL: Interleukin; TNF-: Tumor necrosis factor-alpha; *FGF21*: Fibroblast growth factor 21.

Kyoto Encyclopedia of Genes and Genomes analysis for the potential differential groups

PICRUSt was used to predict the potential functions of microbial genes[17]. In this study, we adopted the PICRUSt2 prediction method to obtain gene function annotations from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Subsequently, statistical analysis of the metagenomic profiles (STAMP) was utilized for differential expression analysis[18] to identify significantly different gene functions among groups. Of note, the KEGG pathway database integrates current knowledge of molecular interaction networks, including biochemical processes such as metabolism, membrane translocation, signal transduction, cell cycle, and conserved subpathways in the same cell lineage.

The obtained findings suggested that the differential pathways ($P < 0.05$) between the *FGF21* + Abx group and the AP group included toluene degradation I (aerobic), toluene degradation III (aerobic), sulfate reduction I, cob(II) acetate a, c-diamine biosynthesis I, and the superpathway of n-acetylceramide degradation (Figure 6A). In addition, the differential pathways ($P < 0.05$) between the db and AP groups included histidine, purine, and pyrimidine biosynthesis, methoxy-13 biosynthesis, methoxy-12 biosynthesis, methoxy-11 biosynthesis, and methoxy-8 biosynthesis (Figure 6B). Furthermore, differential pathways ($P < 0.05$) between the db and *FGF21* + Abx groups included the superpathway of sulfate assimilation and cysteine biosynthesis, superpathway of L-alanine biosynthesis, glyoxylate cycle, tricarboxylic acid cycle I prokaryote, and fatty acid β-oxidation I (Figure 6C). These distinct differentially expressed pathways may provide critical insights into the effects of AP treatment.

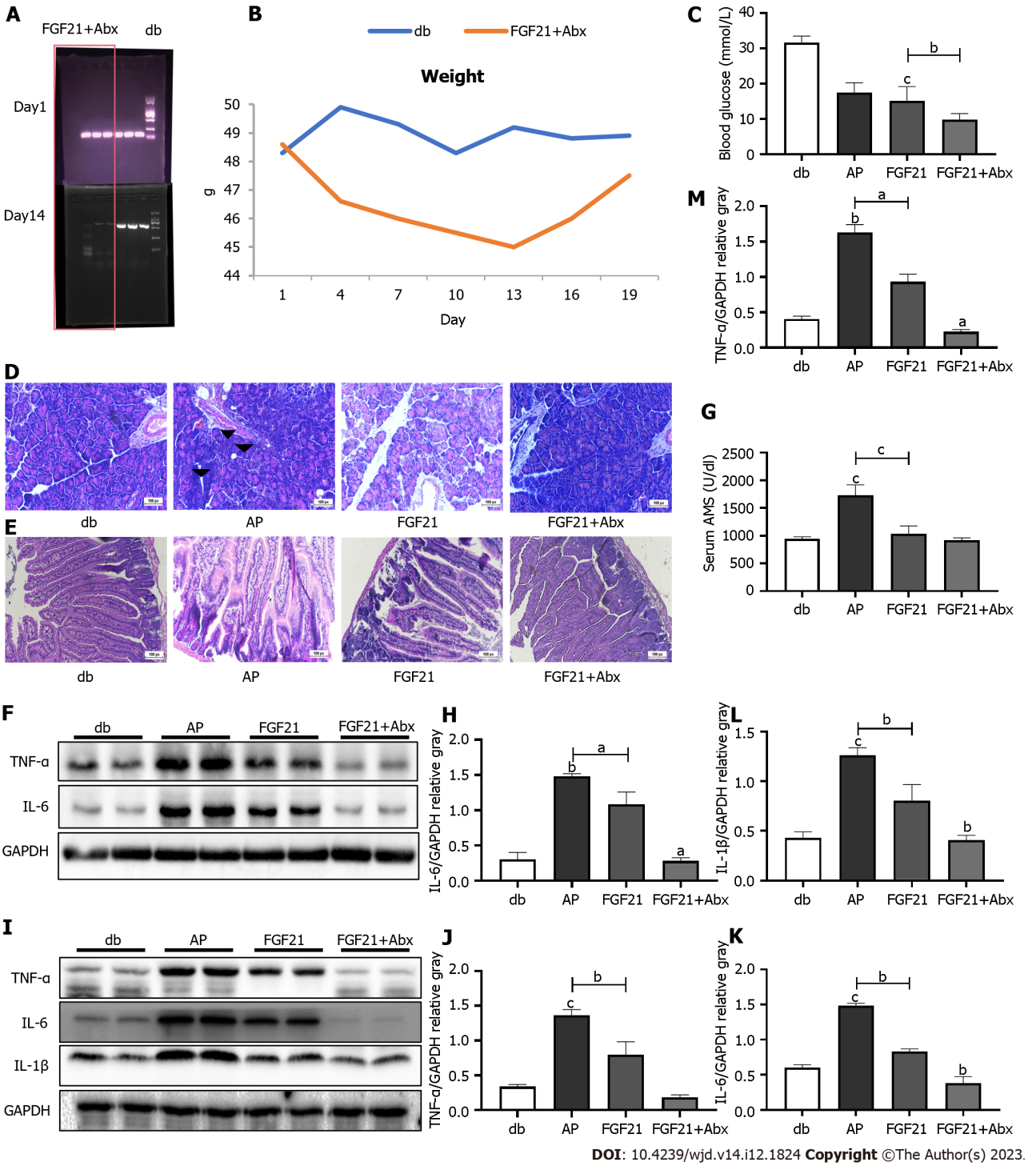

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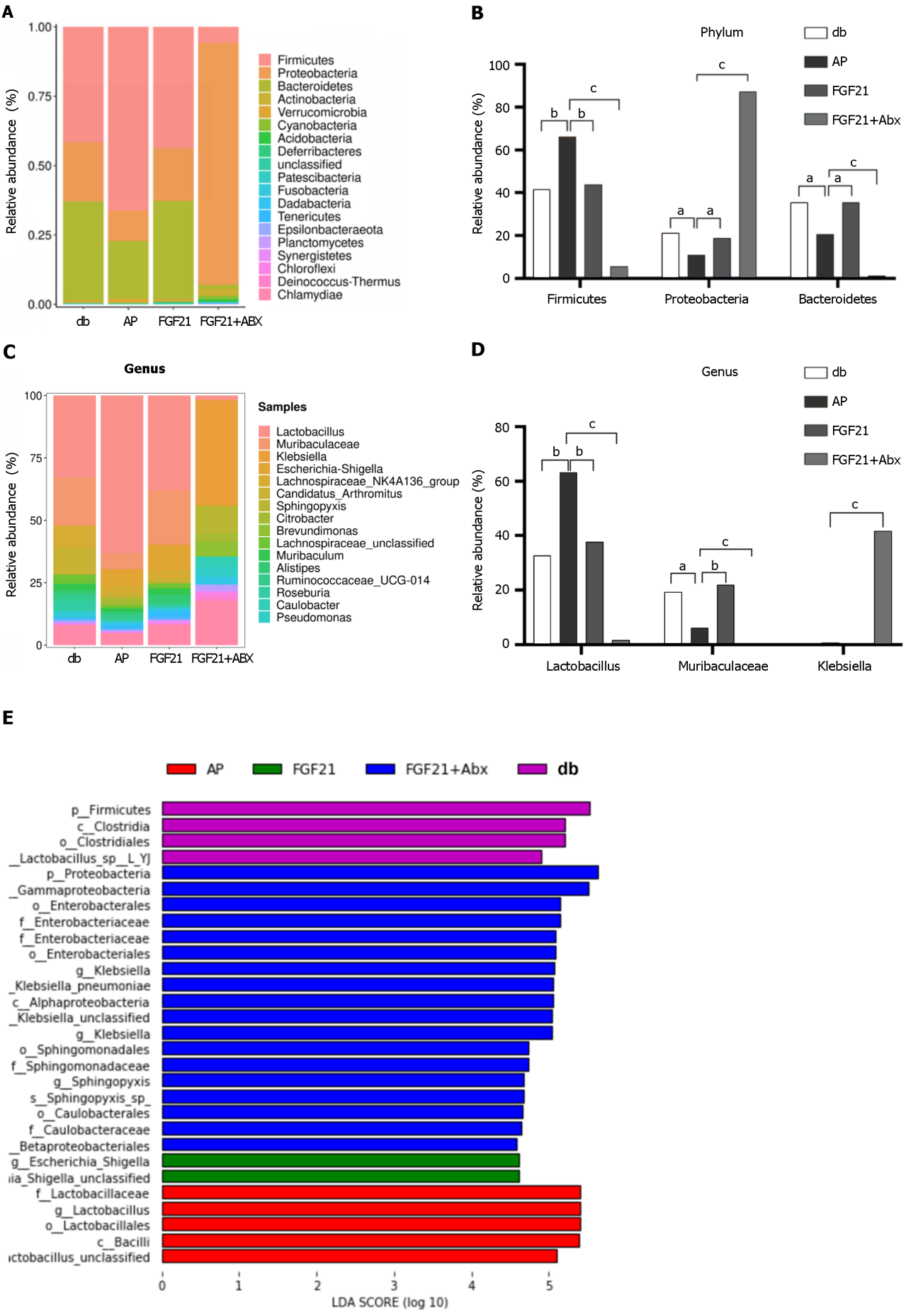
Figure 4 Combined therapy of Abx cocktail and fibroblast growth factor 21 significantly decreases the susceptibility to acute pancreatitis in diabetic mice. A: Verification of the intestinal microbiota removal after feeding antibiotics; B: Following Abx cocktail treatment initiation, the body weight change of mice; C: Serum amylase levels of mice in each group; D: Pathological changes in pancreatic tissues of mice in each group, such as pancreatic edema, extensive intracellular vacuolation, and cellular necrosis (scale bar: 100 μm); E: Histological changes in the small intestine of mice in each group, characterized by tissue edema, increased villus width, and villus damage (scale bar: 100 μm); F-H: Representative immunoblots of inflammatory factors in mouse pancreatic tissue. Expression levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-alpha were quantified using densitometry, with GAPDH as a protein loading control; I-L: Representative immunoblots of inflammatory factors in mouse small intestinal tissue. Expression levels of IL-6, TNF- and IL-1 were quantified using densitometry, with GAPDH as a protein loading control. M: Changes in blood glucose in mice in different groups. Data are presented as mean ± SD, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. AP: Acute pancreatitis; db: Diabetic; IL: Interleukin; TNF-: Tumor necrosis factor-alpha; *FGF21*: Fibroblast growth factor 21.


A
Relative abundance (%)
1.00
0.75
0.50
0.25
0.00
db
AP
FGF21
FGF21+ABX
Firmicutes
Proteobacteria
Bacteroidetes
Actinobacteria
Verrucomicrobia
Cyanobacteria
Acidobacteria
Deferribacteres
unclassified
Patescibacteria
Fusobacteria
Dadabacteria
Tenericutes
Epsilonbacteraeota
Planctomycetes
Synergistetes
Chloroflexi
Deinococcus-Thermus
Chlamydiae
B
Phylum
db
AP
FGF21
FGF21+Abx
Relative abundance (%)
Firmicutes
Proteobacteria
Bacteroidetes
C
Genus
Samples
Relative abundance (%)
Lactobacillus
Muribaculaceae
Klebsiella
Escherichia-Shigella
Lachnospiraceae_NK4A136_group
Candidatus_Arthromitus
Sphingopyxis
Citrobacter
Brevundimonas
Lachnospiraceae_unclassified
Muribaculum
Alistipes
Ruminococcaceae_UCG-014
Roseburia
Caulobacter
Pseudomonas
D
Genus
db
AP
FGF21
FGF21+Abx
Relative abundance (%)
Lactobacillus
Muribaculaceae
Klebsiella
E
AP
FGF21
FGF21+Abx
db
p__Firmicutes
c__Clostridia
o__Clostridiales
_Lactobacillus_sp__L_YJ
p__Proteobacteria
_Gammaproteobacteria
o__Enterobacterales
f__Enterobacteriaceae
f__Enterobacteriaceae
o__Enterobacteriales
g__Klebsiella
_Klebsiella_pneumoniae
c__Alphaproteobacteria
_Klebsiella_unclassified
g__Klebsiella
o__Sphingomonadales
f__Sphingomonadaceae
g__Sphingopyxis
s__Sphingopyxis_sp_
o__Caulobacterales
f__Caulobacteraceae
_Betaproteobacteriales
g__Escherichia_Shigella
ıia_Shigella_unclassified
f__Lactobacillaceae
g__Lactobacillus
o__Lactobacillales
c__Bacilli
ıctobacillus_unclassified
LDA SCORE (log 10)

F

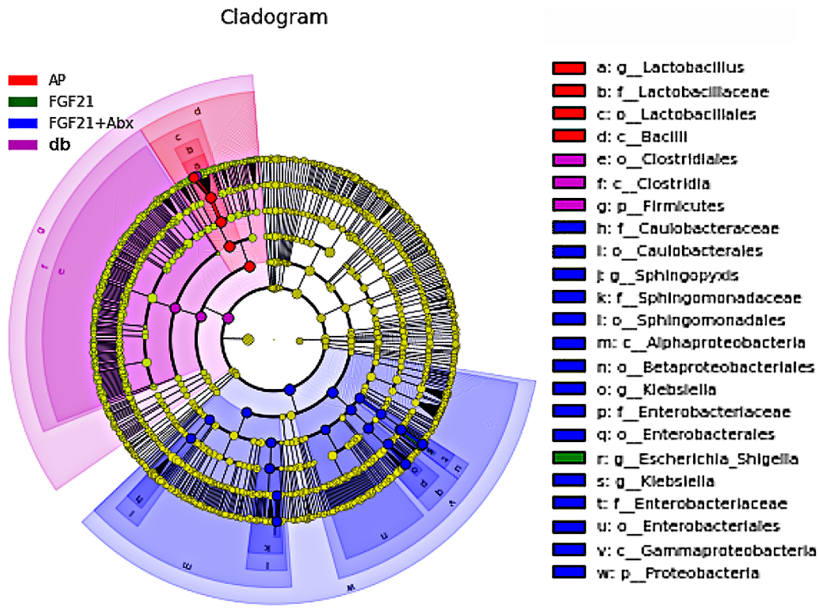


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Figure 5 Abx cocktail combined with fibroblast growth factor 21 treatment alters microbiota in diabetic mice with acute pancreatitis. A: Bar graph of the structural distributions of fecal microbial communities at the phylum level; B: Relative abundance of the dominant phyla; C: Bar graph of the structural distributions of fecal microbial communities at the genus level; D: Relative abundance of the dominant genera; E: Linear discriminant analysis (LDA) scores for the differentially abundant bacterial taxa between each group (LDA > 4.5); F: The phylogenetic tree shows the origins of the microbiota at different taxonomic levels. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. AP: Acute pancreatitis; db: Diabetic; *FGF21*: Fibroblast growth factor 21.

DISCUSSION

AP is a clinically prevalent inflammatory disorder. Previous animal experiments have revealed the potential of *FGF21* to reduce the levels of digestive enzymes (AMS and lipase) in AP mice without affecting protein synthesis[19]. Additionally, *FGF21* has been demonstrated to diminish the release of inflammatory cytokines, such as TNF- and IL-6, indicating a potent anti-inflammatory effect[11]. A previously reported study found that *FGF21* transgenic mice showed significant improvements in pancreatic inflammation and fibrosis in a model of AP induced by ceruletide[20]. In our previous study, it was revealed that *FGF21* exerted protective effects against AP through various mechanisms. These mechanisms included the stimulation of Sirt1 expression, the restoration of impaired mitochondria and lysosomes, the promotion of normal autophagic flux, and the suppression of aberrant expression of digestive enzymes in AP[12]. Furthermore, *FGF21* was found to reduce inflammatory responses, thereby contributing to the amelioration of AP. In the current study, *FGF21* treatment was found to ameliorate histopathological damage in the pancreatic tissues, reduce serum levels of AMS, and diminish levels of pro-inflammatory cytokines (IL-6 and TNF-) in diabetic mice with AP. These results confirmed the potential of *FGF21* in decreasing the susceptibility to AP in diabetic mice. Moreover, both the *FGF21* group and the *FGF21* +Abx group showed a decline in blood glucose levels, indicating that *FGF21* and Abx cocktail therapy effectively alleviated both diabetes and AP in diabetic mice. These findings present a novel and enhanced pharmacological option for diabetic patients complicated by AP.

Notably, a previous comprehensive study was conducted using 16S rRNA sequencing technology to examine the bacteria associated with pancreatitis. It was found that 70% of patients with pancreatitis had various microbial DNA in their bloodstream[21]. The majority of these microbes exhibited similarity to those found in the gastrointestinal tract, suggesting a possible origin from the gut. Hence, we intended to explore whether the intestinal damage caused by diabetes and AP altered the composition of gut microbiota. To address this issue, the present study employed 16S rRNA sequencing technology to analyze the diversity of intestinal microbiota across three groups of mice. The analyses of beta and alpha diversity analyses revealed the differences in gut microbiota composition across the three groups. Notably, the AP group exhibited a significant increase in the abundance of gut microbiota of the AP group, which returned to normal levels after *FGF21* treatment. Prior research has established significant differences in the composition of gut microbiota between obese mice and wild-type mice. Specifically, obese mice showed a notable abundance of bacteria from the phylum *Firmicutes*, while wild-type mice displayed a predominant abundance of bacteria belonging to the phylum *Bacteroidetes*[22]. Furthermore, it has been identified that a decline in the *Bacteroides/Firmicutes* ratio is associated with obesity[23]. Thus, the current study conducted a comparative analysis of bacterial community changes in the mice of three groups, revealing a diminished *Bacteroides/Firmicutes* ratio in the AP group, which was increased after *FGF21* treatment. This finding suggests that AP exacerbates the changes in the gut microbiota of diabetic mice, and both AP and diabetes contribute to the increase in gut microbiota diversity and the decline in the *Bacteroides*/*Firmicutes* ratio. *FGF21* treatment effectively ameliorates the alterations in gut microbiota, thereby facilitating the alleviation of diabetes and AP conditions.

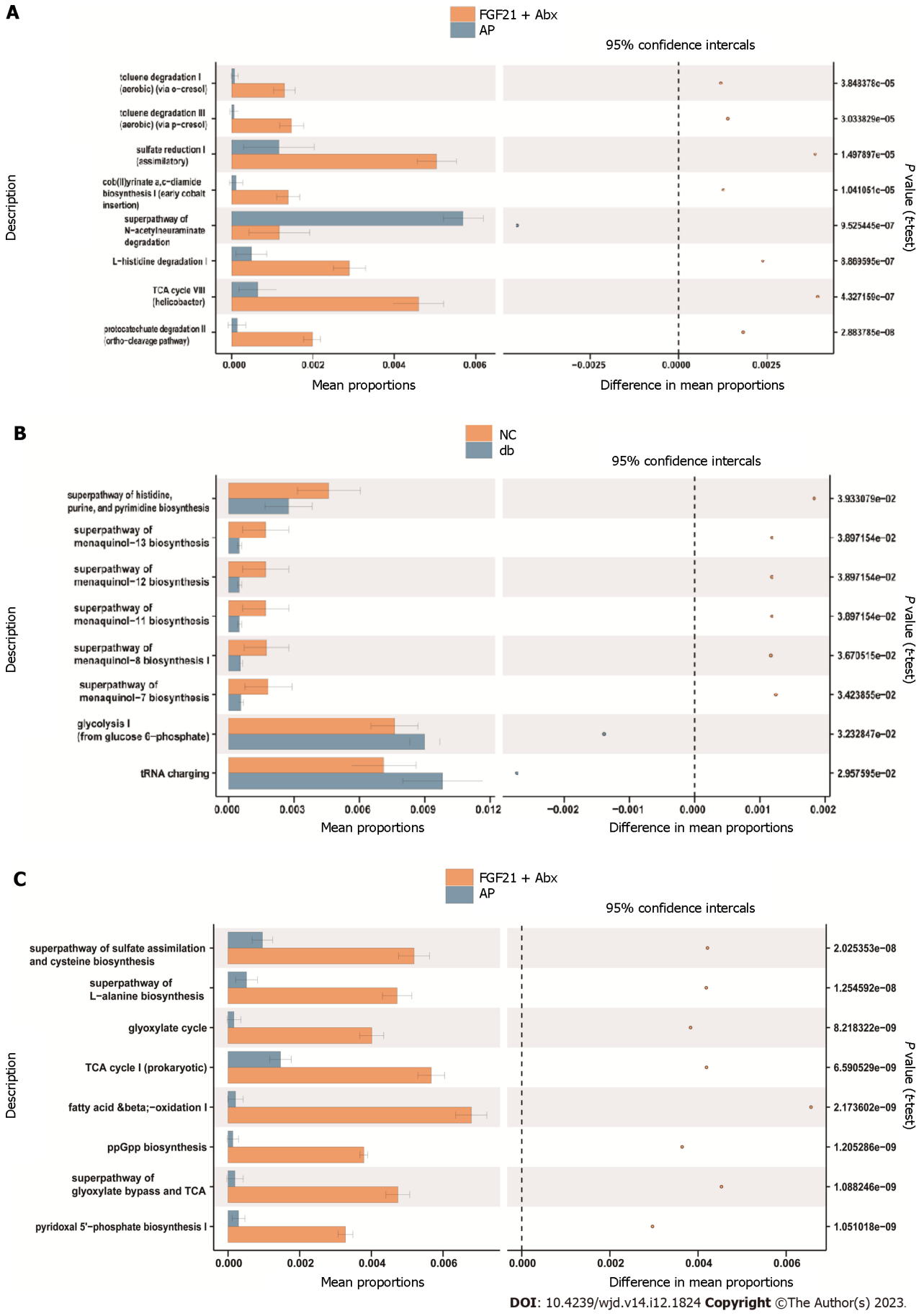

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Figure 6 The significantly different gene functions among groups. A: The differential pathways between the fibroblast growth factor 21 (*FGF21*) + Abx

group and the acute pancreatitis (AP) group; B: The differential pathways between the diabetic (db) and AP groups; C; The differential pathways between the db and *FGF21* + Abx groups. AP: Acute pancreatitis; db: Diabetic; *FGF21*: Fibroblast growth factor 21.

Subsequently, we sought to elaborate on whether the dysbiosis of gut microbiota in diabetic mice with AP was a concomitant phenomenon or an influencing factor in the occurrence and development of AP in diabetic mice. Our investigation also aimed to illuminate whether *FGF21* could ameliorate diabetes and AP condition through the modulation of the gut microbiota. The observations revealed a substantial protective effect of *FGF21* against pancreatitis and intestinal inflammation symptoms. Nonetheless, the effects of Abx on diabetes and systemic inflammation have been a subject of contentious debate in many studies. Some studies have reported the protective effect of Abx against diabetes and systemic inflammation, while in other studies, Abx has been shown to exacerbate the disease[24]. Previous studies have provided evidence demonstrating a reduction in the occurrence of diabetes in mice receiving vancomycin treatment from birth to weaning[25]. Moreover, prior research has also suggested that an Abx cocktail (sulfamethoxazole, trimethoprim, and streptomycin sulfate) lowered the occurrence of diabetes and delayed its onset[26]. The timing of Abx administration, particularly before the onset of diabetes onset in mice, can disrupt the balance of healthy gut microbiota, which in turn could provide an explanation for the increased occurrence of diabetes observed in most studies[27]. Notably, the responses of female and male mice to the same Abx treatments might be significantly different. Therefore, multiple variables, such as Abx type, dosage, administration timing, and the specific animal model, may significantly influence the efficacy of Abx treatment. In this study, the use of an Abx cocktail after the onset of diabetes reduced inflammation in the pancreas and small intestines. The administration of Abx to the mice was beneficial as it facilitated the elimination of harmful bacteria, thereby supporting the protective effect of *FGF21*. Although Abx administration may disrupt the gut microbiota and cause damage to the intestine, *FGF21* was reported to repair intestinal damage and effectively mitigate the adverse effects of Abx treatment on the intestine.

We also observed different microbial compositions in the db, AP, *FGF21*, and *FGF21* + Abx groups. Importantly, the AP group showed a notable increase in the abundance of *Lactobacillus*. *Lactobacillus*, as a probiotic, has been extensively studied. For instance, a recent study has highlighted the role of *Lactobacillus reuteri* in establishing a balanced gut microbiota, thereby mitigating the intestinal permeability damage caused by bacterial translocation. This probiotic also enhances the secretion of IgA in the ileum and colon and increases the populations of $CD4^+$ and $CD8^+$ cells. Thus, *Lactobacillus reuteri* shows promise in ameliorating methotrexate-induced enterocolitis[28]. In this study, the LEfSe analysis revealed that the most abundant taxa in the *FGF21* + Abx group were the phylum *Proteobacteria*, order Enterobacteriales, and family Enterobacteriaceae. The *FGF21* group was predominantly enriched with *Escherichia coli-Shigella*. In contrast, the phylum *Firmicutes*, class Clostridia, and order Clostridiales were most abundant in the db group. These bacteria may serve as targets for the treatment of AP under diabetic conditions. For instance, the deficiency of antimicrobial peptides, which exhibits a negative correlation with the abundance of *Escherichia coli* and *Shigella*, has been linked to intestinal barrier dysfunction and bacterial translocation[29]. *Enterobacter cloacae*, a common type of Bacteroides, can trigger inflammation and promote lipid accumulation, thus the development of metabolic diseases and atherosclerosis[30]. Dysbiosis of various gut probiotics is tightly associated with the progression of diabetes and AP. For instance, the reduction of *Faecalibacterium prausnitzii* abundance has been observed in the gut microbiota of individuals with intestinal diseases and type 2 diabetes has been observed[31,32].

The present study adopted the PICRUSt2 prediction method to obtain gene functional annotations from the KEGG database. In addition, STAMP was employed to perform differential expression analysis and identify gene functions that exhibit significant differences between groups. The identification of differential pathways may provide pivotal insights for AP treatment and unravel the mechanisms whereby gut microbiota modulates the therapeutic effects of *FGF21* on AP under diabetic conditions. Prior research has underscored a decrease in the levels of butyryl-CoA dehydrogenase in patients with diabetes relative to the control group, accompanied by a decrease in butyrate production in the gut microbiota[33]. In our study, *FGF21* + Abx treatment significantly facilitated the sulfate reduction pathway and inhibited the superpathway of n-acetylceramide degradation. These findings suggest significant differences when compared with the AP group and call for further investigation in subsequent studies.

CONCLUSION

This study revealed the potential ability of *FGF21* to enhance the recovery of pancreatic and intestinal damage recovery, reduce blood glucose levels, and modulate the composition of gut microbiota in diabetic mice with AP. Notably, the Abx cocktail therapy further influences the composition of the gut microbiota and enhances the protective effects of *FGF21*. These findings provide new insights into the prevention and treatment of diabetes complicated by AP. However, further investigation is required to elucidate the specific mechanisms by which the gut microbiota affects the protective effects of *FGF21* against AP in diabetic mice.

ARTICLE HIGHLIGHTS

Research background

Fibroblast growth factor 21 (*FGF21*) plays a pivotal role in regulating glucose and lipid metabolism. Acute pancreatitis (AP) is a common inflammatory disease with clinical manifestations. Diabetes exacerbates intestinal permeability and intestinal inflammation, thus leading to the progression to AP. Our previous study indicated that *FGF21* significantly attenuated susceptibility to AP in mice.

Research motivation

Yet, whether *FGF21* similarly protects AP in diabetic mice remains unexplored.

Research objectives

Herein, we were intrigued to investigate the potential protective role of *FGF21* against AP in diabetic mice.

Research methods

In the present study, a mouse model of AP was established in db/db diabetic mice through ceruletide injections. By comparing the differences in AP indicators between diabetic mouse group (db), ceruletide-induced AP model group (AP), *FGF21* treatment group (*FGF21*), and *FGF21* combined with an antibiotic (Abx) cocktail treatment group (*FGF21* + Abx), we investigated the protective effect of recombinant *FGF21* protein and investigated whether *FGF21* plays its role in the treatment of diabetic mice with AP by modulating the gut microbiota.

Research results

FGF21 notably diminished the levels of serum amylase, inflammatory factors and the histological evidence of inflammation in the pancreas and the small intestine in diabetic mice with AP. *FGF21* also significantly altered the composition of the gut microbiota, reestablishing the *Bacteroidetes/Firmicutes* ratio. Upon treatment with an Abx cocktail to deplete the gut microbiota, the *FGF21* + Abx group showed superior protective effect. The gut microbiota composition across different groups was further characterized, and a differential expression analysis of gene functions was undertaken using the PICRUSt2 prediction method. These findings suggested that *FGF21* could potentially confer therapeutic effects on diabetic mice with AP by modulating the sulfate reduction I pathway and the superpathway of n-acetylceramide degradation in the gut microbiota.

Research conclusions

This study reveals the potential of *FGF21* in improving pancreatic and intestinal damage recovery, reducing blood glucose levels, and reshaping gut microbiota composition in diabetic mice with AP. Notably, the protective effects of *FGF21* are augmented when combined with the Abx cocktail. These findings provide new insights into the prevention and treatment of diabetes complicated by AP.

Research perspectives

Further investigation is required to elucidate the specific mechanisms by which the gut microbiota affects the protective effects of *FGF21* against AP in diabetic mice.

FOOTNOTES

Author contributions: Sun QY, Wang XY and Huang ZP contributed equally to this work; Gong FH and Huang XW conceived the experiments, analyzed the data; Sun QY, Wang XY, Huang ZP, Song J and Zheng ED performed experiments, coordinated the study and oversaw all experiments, revised the paper; all authors discussed the results and commented on the manuscript.

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Institutional animal care and use committee statement: The study was reviewed and approved by the Ethics Committee of the Laboratory Animal of Wenzhou Medical University Institutional Review Board (Approval No.xmsq2023-0426).

Conflict-of-interest statement: The author declare that there is no conflict of interest regarding the publication of this manuscript.

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Abstract

BACKGROUND

Some studies have directed towards an association between diabetes mellitus (DM) and prostate cancer (PCa); however, this specific relationship remains inconclusive. In recent years, Mendelian randomization (MR) has become a widely used analytical method for inferring epidemiological causes.

AIM

To investigated the potential relationship between DM and PCa using MR.

METHODS

We downloaded relevant data on "diabetes" and "PCa" from the IEU OpenGWAS project database, performed three different methods to conduct MR, and carried out sensitivity analysis for verification.

RESULTS

The results indicated that DM was an independent risk factor for PCa. The odds ratio (OR) values obtained using the inverse variance weighted method in this study were as follows: OR = 1.018 (95% confidence interval: 1.004-1.032), P = 0.014.

CONCLUSION

We found that DM could increase the incidence rate of PCa.

Key Words: Prostate cancer; Diabetes mellitus; Mendelian randomization analysis; Risk factors; Genome-wide association study

Core Tip: Diabetes mellitus (DM) is a chronic metabolic disease caused by many factors. Prostate cancer (PCa) is a common malignant tumor in men and is the second leading cause of cancer death. The Mendelian randomization (MR) method uses genetic variation as an instrumental variable to detect and quantify causal relationships, which can avoid the impact of confounding factors on the accuracy of the research results. This makes it more reliable than observational study or even randomized controlled trial. This study aimed to clarify the relationship between DM and PCa using MR analysis. Through MR analysis of a large sample with three different methods, this study found that DM was an independent risk factor for PCa, providing new directions for the prevention and treatment of PCa.

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INTRODUCTION

Diabetes mellitus (DM) is a major chronic disease worldwide, causing huge burden and harm to patients and their families[1,2]. Currently, prevention is the primary treatment for DM. Its occurrence and development are related to many factors such as diet, lifestyle, and environment[3-5]. Prostate cancer (PCa) is one of the most common cancers worldwide and the second most common cancer in men[6]. In recent years, the diagnostic and treatment modalitites for PCa have greatly improved. However, its incidence rate is steadily increasing, and the age of onset has been decreasing[7]. Currently, the recognized high-risk factors for PCa include age, family history, and ethnic background[8]. Some exogenous factors (such as obesity, diabetes, metabolic syndrome, and dietary factors) are also reportedly associated with PCa; however, this remains inconclusive[9,10]. Given the huge burden of PCa on human health, it is important to identify relevant high-risk factors for its prevention and treatment. This study aimed to investigate the effects of DM on PCa.

Mendelian randomization (MR) is a data analysis method that has been widely used in inferring epidemiological etiology in recent years. It can strengthen causal inference using genetic variation as an instrumental variable (IV). This analysis method is based on the Mendelian inheritance law, so the association between genes and diseases is free from the interference of the postpartum environment, socioeconomic status, behavioral factors, and other common confounding factors, and the resulting causal sequence is reasonable and closer to a real situation[11]. This research method is conceptually similar to a randomized controlled study in which genetic variations are randomly assigned during gamete formation before being interfered with by any confounding factors and are evenly distributed within the population. Alleles are fixed among individuals and do not change with disease occurrence or development. Therefore, the causal inference obtained from MR is not easily affected by residual confounding factors or reverse causality[12-14]. In this study, we obtained sufficient genome-wide association study (GWAS) data from relevant databases and performed a study to assess the impact of DM on PCa based on MR.

MATERIALS AND METHODS

Study design

The premise of MR analysis is that IVs must meet three preconditions: (1) Exposure correlation (correlation hypothesis); (2) no common cause with the outcome (independence assumption); and (3) outcome related only through exposure (excluding restriction assumptions). Based on these criteria, we performed MR to explore the causal relationship between DM and PCa. The entire process of the study primarily included five steps: (1) Fetching exposure factor GWAS data, (2) sifting appropriate IVs, (3) inputting the outcome GWAS data and drawing single nucleotide polymorphisms (SNPs) of the above IVs, (4) preprocessing the exposure factor and outcome GWAS data to ensure consistency in format, and (5) conducting MR and sensitivity analysis.

Data source

SNPs associated with DM were downloaded from the IEU OpenGWAS project database, using phenotype "DM" in this study. Its GWAS ID was "ukb-a-306," the sample size was 336473 and included 10894596 SNPs. The pooled data for prostate cancer was obtained from the GWAS phenotyped "PCa" (GWAS ID: ukb-a-57; sample size: 337159; SNPs' number: 10894596), which was also derived from the IEU OpenGWAS project database. The research data were open and transparent, and could be downloaded directly from relevant websites; therefore, no additional ethical declaration or consent was required.

Selection of IV

We screened SNPs under the genome-wide significance threshold ($P < 5 \times 10^{-8}$) related to exposure interest as potential SNPs, visualized the results of the correlation analysis, and generated Manhattan plots. In both graphs, the red lines represented the filtering conditions of $P < 5 \times 10^{-8}$ (Figure 1). Next, we used a clump function ($r^2 = 0.001$, kb = 10000) to

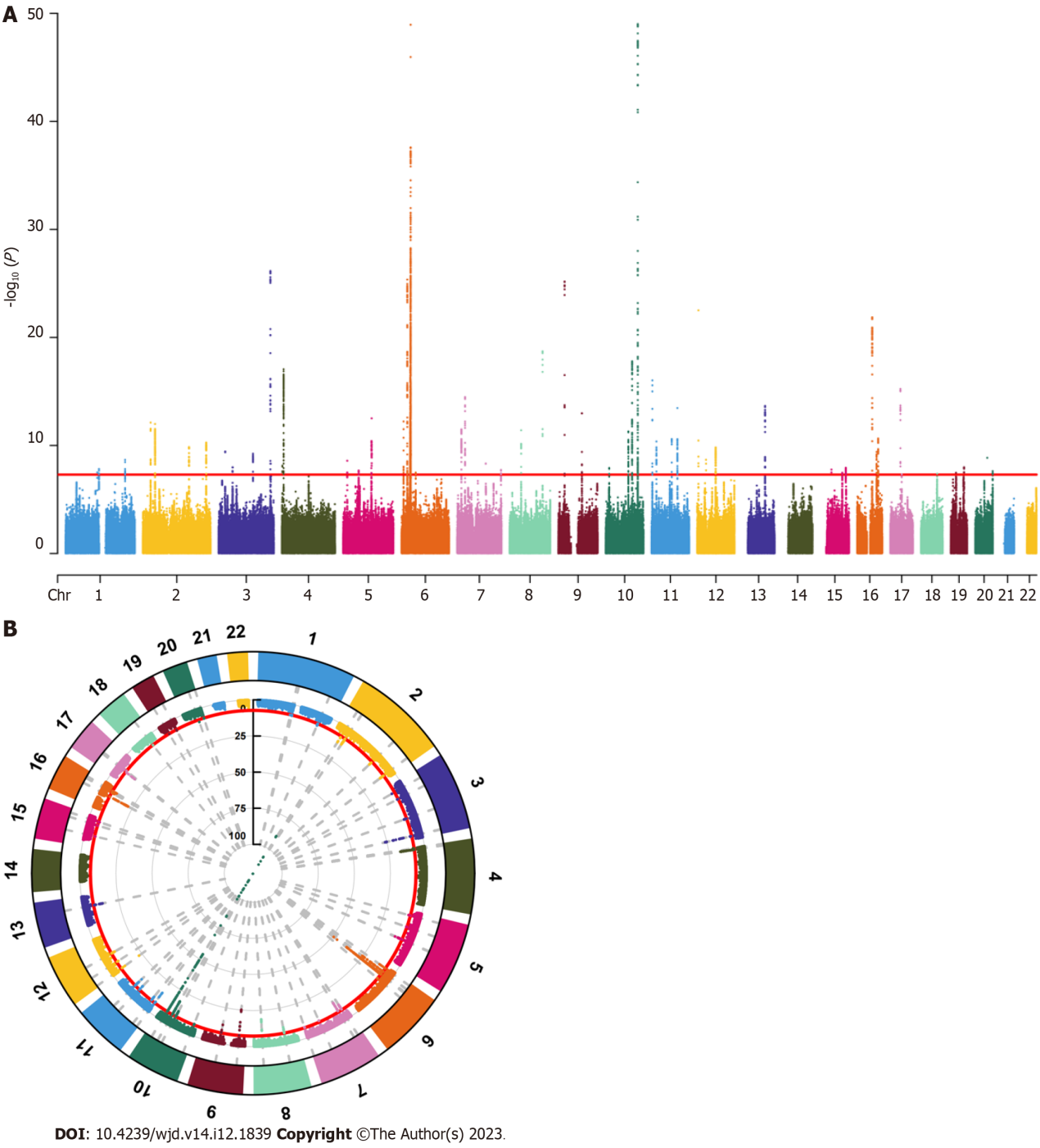

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Figure 1 Selection of instrumental variables. A: Manhattan plot (line graph); B: Manhattan plot (cyclic graph). The red lines represent the filtering conditions of $P < 5 \times 10^{-8}$.

eliminate linkage disequilibrium between the selected SNPs. Further F-statistics were employed to evaluate the effect of weak IVs[15,16], when the F-statistic was less than 10, the genetic variation was considered a weak IV and might have caused bias in the research results. After removing the weak IVs, we created a comprehensive web-based genotype-phenotype association database ("phenoScanner") to further investigate whether the remaining SNPs were related to potential risk factors for PCa, such as long-term bedridden diseases and serious diseases[17,18]. For SNPs associated with confounding factors, we conducted manual screening at the genome-wide significance level $P < 5 \times 10^{-8}$ to remove them. After obtaining the remaining SNPs, relevant adjustments were made to ensure that the impact of the IVs on exposure and outcomes corresponded to the same effector alleles. Finally, we removed SNPs with palindromic sequences whose orientation could not be determined and incompatible SNPs, and used the remaining SNPs as IVs for MR analysis.

MR analysis

To avoid the impact of potential pleiotropy, we employed three different MR methods to assess the causal effect between DM and PCa: The inverse variance weighted (IVW), weighted median, and weighted mode methods. Among them, the

result of the IVW method was considered to be the main result, as the IVW method assumed that all IVs were valid[19-21]. The results of the MR analysis were visualized as the corresponding plots.

Sensitivity analysis

To demonstrate the reliability of the results, we conducted a sensitivity analysis to evaluate pleiotropy and heterogeneity. First, Cochran's Q test was performed to detect potential heterogeneity. When $P < 0.05$, Cochran's Q statistic evaluated the heterogeneity between genetic variation and the heterogeneity that considered. The results were visualized as corresponding funnel plots. Subsequently, we performed MR-Egger intercept tests to evaluate the horizontal pleiotropy, when $P < 0.05$, there was pleiotropy in the result. If pleiotropy occurred, further analysis and identification of the source of pleiotropy were conducted through MR-PRESSO analysis. Finally, leave-one-out analysis was conducted to evaluate whether the causal relationship obtained in the study depended on or leaned towards a single SNP[22].

RESULTS

IVs

Through this screening process, we ultimately screened 49 SNPs as IVs for MR analysis. The F-statistic of all IVs was > 10, indicating the absence of weak IVs bias (Table 1).

MR analysis

MR is a data analysis technique used in epidemiological studies to evaluate causal inferences. It uses genetic variation as the IVs in nonexperimental data to estimate the causal relationship between the exposure factors and outcomes of interest. Using the fixed nature of genes and Mendelian laws of inheritance, the MR analysis results were not affected by common confounding factors such as the postnatal environment, socio-economic factors, and behavioral habits. The causal relationship derived from MR is more reasonable and reliable.

The results of all three MR methods used for analysis revealed that DM was positively correlated with the incidence of PCa. Specifically, using the IVW method as the main analysis method, the OR values obtained in this study were OR = 1.018 (95%CI: 1.004-1.032), $P = 0.014$. Based on these results, we plotted corresponding scatter and forest plots (Figure 2).

Sensitivity analysis

Finally, to verify the reliability of the results further, we performed a sensitivity analysis to examine the heterogeneity and pleiotropy of our conclusions. Cochran's Q test results showed no heterogeneity in the IVs included in the study ($P > 0.05$), and the corresponding funnel plot was shown in Figure 3. MR-PRESSO analysis did not find significant pleiotropy in the conclusion nor did it screen for SNPs with outliers ($P > 0.05$). The test results of the leave-one-out method indicated that the causal relationship between DM and PCa did not depend on or lean towards any single SNP.

DISCUSSION

DM is a chronic metabolic disease caused by many factors[23,24]. Many studies indicate that the best treatment is to prevent the occurrence of diabetes by maintaining a healthy weight and increasing physical activity[25-27]. PCa is a common malignant tumor in men and is the second leading cause of cancer death[28]. Because of its inconspicuous development, most PCa patients are undiagnosed in the early stages[29]. In addition, because of the heterogeneity of tumor cells, approximately 90% of patients present with local or systemic metastasis at the time of diagnosis, losing the opportunity for radical surgery[30,31]. Therefore, early prevention of PCa and implementation of effective intervention measures are particularly important and can significantly improve patient prognosis.

The relationship between DM and PCa has long been the focus of research. Some scholars believe that DM is a protective factor for patients with PCa, whereas others believe that it is a high-risk factor for PCa. Evidence supporting both hypotheses has been reported; thus far, no conclusions have been reached. Epidemiological investigations have shown that the risk of cancer (including liver cancer, pancreatic cancer, colorectal cancer, breast cancer, and endometrial cancer) in patients with DM increases significantly, and the risk of cancer mortality also increases significantly[32]. In a 14 year cross-sectional study, Saewai *et al*[33] found that the long-term risk of PCa was significantly increased in patients with DM. Other studies have shown that obesity and DM are independent risk factors for PCa and may have synergistic effects, further increasing the risk of invasive PCa[34,35]. Another study also found that advanced PCa with DM was associated with a worse prognosis and a greater risk of metastasis[36]. Sánchez-Maldonado *et al*[37] confirmed that functional type 2 DM-related mutations may affect the risk of PCa at the genetic level. Kingshott *et al*[38] also found that DM could directly affect regulatory growth factors related to cancer, and that changing living habits might significantly reduce the risk of prostate and other cancers. Relevant research has shown that patients with DM have a higher risk of recurrence[39]. However, compared with other drugs, the use of metformin in patients with DM can significantly reduce the risk of new-onset PCa, which also proves that intervention in the development of DM has a positive impact on the prevention and treatment of PCa[40,41].

The MR method uses genetic variation as an IV to detect and quantify causal relationships, which can avoid the impact of confounding factors on the accuracy of the research results. This makes MR study more reliable than observational study or even randomized controlled trial. This study aimed to clarify the relationship between DM and PCa using MR.

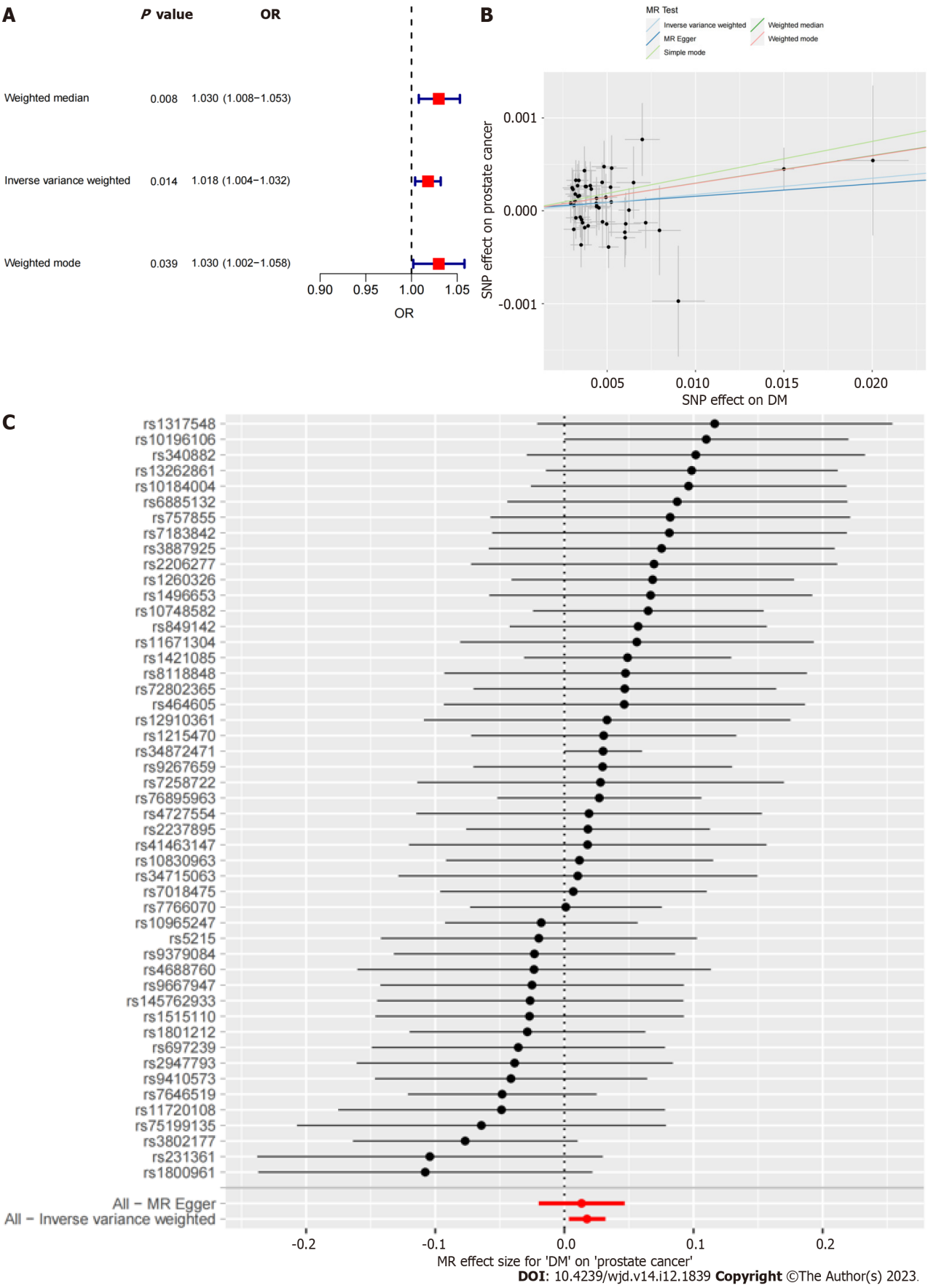

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Figure 2 Mendelian randomization analysis. A: Forest plot of three mendelian randomization methods; B: Scatter plot; C: Forest plot for each single nucleotide polymorphism. DM: Diabetes mellitus; MR: Mendelian randomization; SNP: Single nucleotide polymorphism.

Table 1 49 Single nucleotide polymorphisms selected

SNP	Outcome	Exposure	*P* val. exposure
rs10184004	PCa	DM	1.42E-10
rs10196106	PCa	DM	1.02E-12
rs10748582	PCa	DM	1.61E-18
rs10830963	PCa	DM	3.45E-14
rs10965247	PCa	DM	6.65E-26
rs11671304	PCa	DM	1.03E-08
rs11720108	PCa	DM	5.75E-10
rs1215470	PCa	DM	2.17E-14
rs1260326	PCa	DM	7.61E-13
rs12910361	PCa	DM	3.34E-08
rs1317548	PCa	DM	1.19E-08
rs13262861	PCa	DM	3.90E-12
rs1421085	PCa	DM	1.36E-22
rs145762933	PCa	DM	3.85E-11
rs1496653	PCa	DM	3.66E-10
rs1515110	PCa	DM	5.35E-11
rs1800961	PCa	DM	1.40E-09
rs1801212	PCa	DM	8.72E-18
rs2206277	PCa	DM	3.23E-08
rs2237895	PCa	DM	9.39E-17
rs231361	PCa	DM	4.68E-09
rs2947793	PCa	DM	1.52E-10
rs340882	PCa	DM	2.14E-09
rs34715063	PCa	DM	1.72E-08
rs34872471	PCa	DM	3.60E-151
rs3802177	PCa	DM	1.90E-19
rs3887925	PCa	DM	4.88E-09
rs41463147	PCa	DM	1.51E-08
rs464605	PCa	DM	2.08E-08
rs4688760	PCa	DM	1.03E-08
rs4727554	PCa	DM	4.59E-09
rs5215	PCa	DM	1.49E-10
rs6885132	PCa	DM	2.60E-09
rs697239	PCa	DM	5.00E-12
rs7018475	PCa	DM	3.02E-14
rs7183842	PCa	DM	1.15E-08
rs7258722	PCa	DM	3.37E-08
rs72802365	PCa	DM	2.26E-11
rs75199135	PCa	DM	4.11E-08
rs757855	PCa	DM	1.83E-08
rs7646519	PCa	DM	7.02E-27

rs76895963	PCa	DM	3.07E-23
rs7766070	PCa	DM	4.38E-26
rs8118848	PCa	DM	2.38E-08
rs849142	PCa	DM	3.31E-15
rs9267659	PCa	DM	4.84E-15
rs9379084	PCa	DM	6.03E-13
rs9410573	PCa	DM	1.04E-13
rs9667947	PCa	DM	2.55E-11

DM: Diabetes mellitus; SNP: Single nucleotide polymorphism; PCa: Prostate cancer.

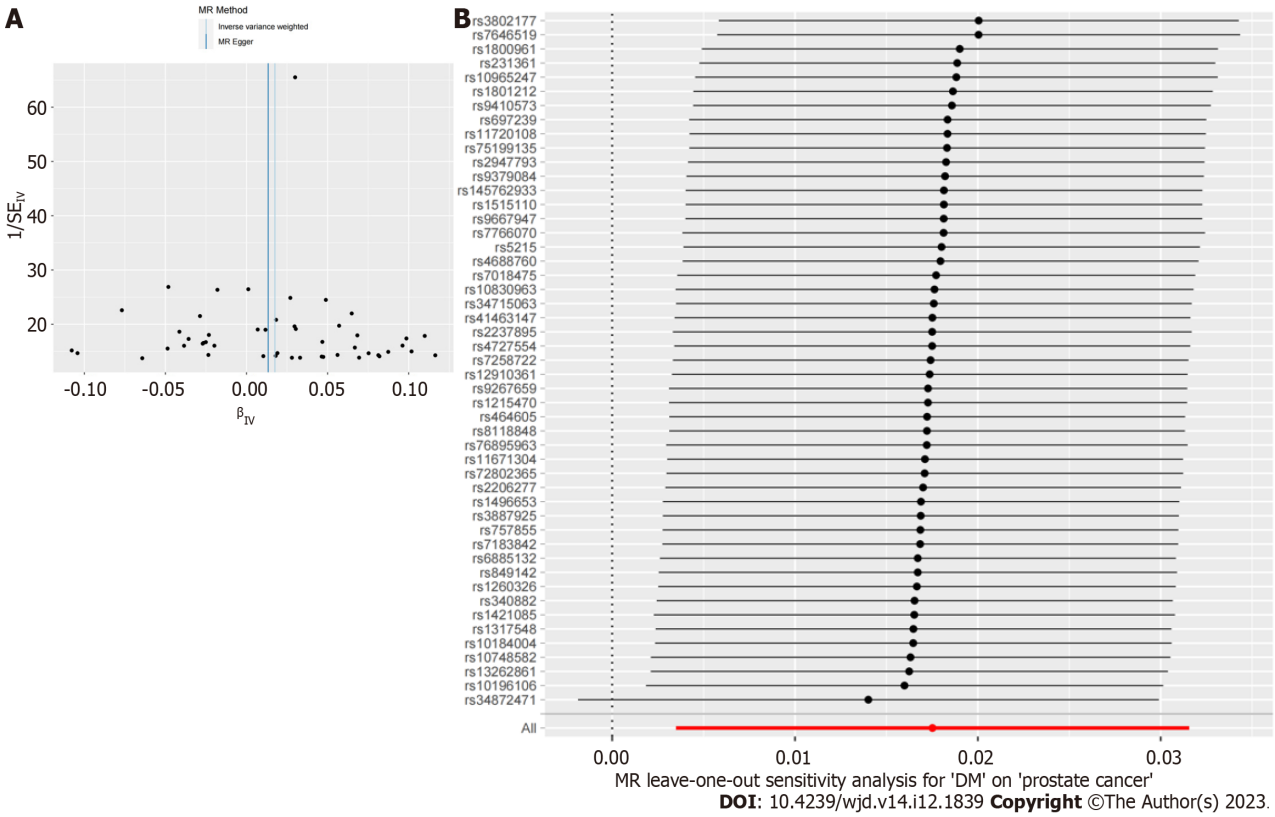

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Figure 3 Sensitivity analysis. A: Funnel plot; B: The result of leave-one-out method. DM: Diabetes mellitus; IV: Instrumental variable.

Compared to the previous observational study, this study explored the potential causal relationship between DM and PCa using three different MR methods. Through MR analysis, we found that DM was a high-risk factor for PCa, which was consistent with previous clinical experience and the results of numerous studies. The results of the sensitivity analysis validation also indicated that the obtained results were reliable. The results of the three MR methods showed that DM increased the risk of PCa. Based on the results of this study, we could conduct early clinical screening of high-risk (DM) populations, control their weight, and strengthen their exercises to further reduce the incidence rate of PCa. DM has already been regarded as a high-risk factor for PCa in some clinical guidelines and academic researches, and our results could also provide a theoretical basis.

This study had some limitations. First, the GWAS dataset obtained in our study was from the same population (European) and could be supplemented in subsequent studies to further expand the coverage of the research results. Second, DM and PCa could be divided into many subtypes, and future research should further explore the relationship between these subtypes. Third, we excluded only SNPs associated with known confounding factors, and future research was needed to further exclude other unknown confounding factors. Finally, it should be noted that the results of MR research could only partially explain the causal effect of DM on PCa. Other methods were needed to prove this result in the future.

CONCLUSION

Through MR analysis of a large sample, this study found that DM was an independent risk factor for PCa, providing new directions for the prevention and treatment of PCa.

ARTICLE HIGHLIGHTS

Research background

Some studies have shown a relationship between diabetes mellitus (DM) and prostate cancer (PCa); however, this specific relationship remains inconclusive.

Research motivation

Mendelian randomization (MR) has been a widely used analytical method in recent years for inferring epidemiological causes. We believe that MR can explain the causal relationship between DM and PCa.

Research objectives

Find the causal relationship between DM and PCa.

Research methods

We downloaded the relevant data from a public database, used three different MR methods, and conducted a sensitivity analysis for validation.

Research results

These results indicated that DM was an independent risk factor for PCa. The odds ratio (OR) values obtained using the inverse variance weighted method in this study were as follows: OR = 1.018 (95% confidence interval: 1.004-1.032), P = 0.014.

Research conclusions

Through MR analysis of a large sample, this study found that DM was an independent risk factor for PCa, providing new directions for the prevention and treatment of PCa.

Research perspectives

This study investigated the potential relationship between DM and PCa using MR.

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FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Atorvastatin ameliorated myocardial fibrosis in db/db mice by inhibiting oxidative stress and modulating macrophage polarization

Xian-Min Song, Meng-Nan Zhao, Gui-Zhi Li, Na Li, Ting Wang, Hong Zhou

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Abstract

BACKGROUND

People with diabetes mellitus (DM) suffer from multiple chronic complications due to sustained hyperglycemia, especially diabetic cardiomyopathy (DCM). Oxidative stress and inflammatory cells play crucial roles in the occurrence and progression of myocardial remodeling. Macrophages polarize to two distinct phenotypes: M1 and M2, and such plasticity in phenotypes provide macrophages various biological functions.

AIM

To investigate the effect of atorvastatin on cardiac function of DCM in db/db mice and its underlying mechanisms.

METHODS

DCM mouse models were established and randomly divided into DM, atorvastatin, and metformin groups. C57BL/6 mice were used as the control. Cardiac function was evaluated by echocardiography. Hematoxylin and eosin and Masson staining was used to examine the morphology and collagen fibers in myocardial tissues. The expression of transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), interleukin-1 β (IL-1β),M1 macrophages ($iNOS^+$), and M2 macrophages ($CD206^+$) were demonstrated by immunohistochemistry and immunofluorescence staining. The levels of TGF-β1, IL-1β, and TNF-α were detected by ELISA and real-time quantitative polymerase chain reaction. Malondialdehyde (MDA) concentrations and superoxide dismutase (SOD) activities were also measured.

RESULTS

Treatment with atorvastatin alleviated cardiac dysfunction and decreased db/db mice. The broken myocardial fibers and deposition of collagen in the myocardial interstitium were relieved especially by atorvastatin treatment. Atorvastatin also reduced the levels of serum lactate dehydrogenase, creatine kinase isoenzyme, and troponin; lowered the levels of TGF-β1, TNF-α and IL-1β in serum and myocardium; decreased the concentration of MDA and increased SOD activity in myocardium of db/db mice; inhibited M1 macrophages; and promoted M2 macrophages.

CONCLUSION

Administration of atorvastatin attenuates myocardial fibrosis in db/db mice, which may be associated with the antioxidative stress and anti-inflammatory effects of atorvastatin on diabetic myocardium through modulating macrophage polarization.

Key Words: Atorvastatin; Diabetic cardiomyopathy; Myocardial fibrosis; Macrophage polarization; Inflammation; Oxidative stress

Core Tip: The occurrence and development of diabetic cardiomyopathy are accompanied by a few pathological mechanisms. The present study showed that atorvastatin had antioxidant properties on diabetic hearts. Cardiac tissues include many resident macrophages. In high glucose conditions, macrophages can upregulate glucose uptake and utilization and enhance the production of inflammatory cytokines. Dysregulation of macrophages between M1 and M2 phenotypes causes excessive inflammation and cardiac injury. Our study suggests that administration of atorvastatin attenuates myocardial fibrosis in db/db mice, which may be associated with the antioxidative stress and anti-inflammatory effects of atorvastatin on diabetic myocardium through modulating macrophage polarization.

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INTRODUCTION

The prevalence of diabetes mellitus (DM) is rising around the world, and is becoming a significant concern for global health. People with DM suffer from multiple chronic complications due to sustained hyperglycemia, especially diabetic cardiomyopathy (DCM). DCM is defined as myocardial structural abnormality and cardiac dysfunction, characterized by early diastolic dysfunction and further obstacle of systolic function, which ultimately leads to refractory heart failure (HF), independent of hypertension, coronary artery disease, or heart valvular disease[1]. Autophagy dysregulation, abnormal mitochondrial energetics, oxidative stress, inflammation, impaired calcium homeostasis, and activation of the renin–angiotensin–aldosterone system are all involved in the pathogenesis of DCM[2]. The pathological changes of DCM mainly contain cardiomyocyte hypertrophy, apoptosis, and myocardial fibrosis. Cardiac fibrosis is a major contributor to cardiac dysfunction, which ultimately increases the incidence of hospitalization due to HF and the mortality in patients with DM. However, there is currently no specific treatment for DCM at the early stage.

DM is a mild, chronic inflammatory condition characterized by the excessive secretion of proinflammatory cytokines, which can lead to cardiovascular complications[3]. Oxidative stress and inflammatory cells play crucial roles in the occurrence and progression of myocardium remodeling. High-glucose-induced oxidative stress can induce cardiac fibroblasts switching to a profibrotic phenotype that leads to cardiac fibrosis[4-6]. Cardiac fibrosis is mediated by a number of inflammatory cytokines, such as transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). As a profibrotic regulator, TGF-β1 regulates fibroblast to myofibroblast transformation[7]. In our previous study, we observed an increase in Smad2/3 phosphorylation in the hearts of diabetic rats, coupled with elevated mRNA and protein levels of TGF-β1. JNK and Smad2/3 may serve as downstream signaling molecules within the RhoA/ROCK pathway and play a role in the development of myocardial fibrosis in type 2 DM (T2DM) rats[8]. Che *et al*[9] also discovered that inhibiting the TGF-β1/Smads signaling pathway significantly ameliorated cardiac dysfunction and reduced collagen production in DM mice. Inhibition of TNF-α has been shown to reduce cardiac fibrosis and improve cardiac function, contributing to the amelioration of DCM[10,11]. Hsuan *et al*[2] demonstrated that inhibiting the p38 mitogen-activated protein kinase stress pathway decreased inflammatory cytokines such as TNF-α and IL-1β in diabetic hearts, thus improving left ventricular dysfunction in DCM. Similarly, IL-1β plays a significant role in the pathophysiology of DCM, and targeting the NACHT, LRR, and PYD domains-containing protein 3 (NLRP3)/IL-1β pathway may prove effective in alleviating this disease burden. Incorporating IL-1β inhibition alongside statin therapy may offer added cardiovascular protection benefits[3,12]. Inflammatory cells and macrophages belong to the family of mononuclear phagocytes and play vital roles in immune responses, homeostasis, tissue damage, and restoration[13]. Macrophages

polarize to two distinct phenotypes: M1 and M2, and such plasticity in phenotypes provide macrophages various biological functions. M1 and M2 macrophages are closely associated with inflammatory responses; M1 macrophages are mainly involved in proinflammatory responses through secreting various proinflammatory mediators, leading to tissue injury. For example, a hyperglycemic state triggers aggregation of M1 macrophages, while proinflammatory cytokines such as TNF-α, IL-1β and TGF-β1 are elevated, stimulating myocardial fibrosis[14,15]. M2 macrophages exert anti-inflammatory effects, contributing to tissue repair[16]. It has been shown that the imbalance of the M1/M2 ratio accelerates the development of DCM, and the regulation on macrophage polarization can improve cardiac dysfunction in DCM mice[17,18].

Metformin is a first-line glycemic control drug that can decrease glycogen output and increase peripheral tissue uptake of glucose, thus ameliorating insulin resistance[19]. It is reported that metformin protects against DCM through attenuating cardiac apoptosis and fibrosis[20,21]. Therefore, metformin has been commonly used as the positive drug control for T2DM[22]. Statins are lipid-lowering drugs, possessing anti-inflammatory and antioxidative effects. This study aimed to investigate the effect of atorvastatin on cardiac function of DCM in db/db mice and its underlying mechanisms.

MATERIALS AND METHODS

Animals and treatment

Six-week-old male db/db mice and C57BL/6 mice were purchased from the HFK Bio-Technology Co. Ltd. (Beijing, China) (Approval No. SCXK 2020-0004). All procedures were approved by the Animal Experimental Ethics Committee of the Second Hospital of Hebei Medical University and the Animal Health Care Guidelines to minimize animal suffering. All mice were housed in standard cages (5 mice/cage) and were fed in a room with moderate temperature (22 ± 2°C), appropriate humidity (55% ± 5%), a 12/12 h light/dark cycle, with chow diet and water *ad libitum*. At 8 wk of age, blood glucose levels were measured from the tail vein using a portable glucometer (Accu-Chek Active; Roche Diagnostics, Mannheim, Germany). The db/db mice were considered to have T2DM if their blood glucose level was ≥ 16.7 mmol/L [23]. The diabetic mice were randomly divided into three groups: DM: db/db mice received daily oral gavage of sterilized water; atorvastatin group (DM + ATO): db/db mice received daily oral gavage of 10 mg/kg/d atorvastatin; metformin group (DM + MET): db/db mice received daily oral gavage of 200 mg/kg metformin. The C57BL/6 mice were used as the control group (CON). Each group contained five mice. Metformin hydrochloride tablets were purchased from Sino-American Shanghai Squibb Pharmaceutical Co. Ltd. (Shanghai, China), and atorvastatin calcium was purchased from Pfizer (New York, USA). Both drugs were dissolved in sterilized water and administrated through gastric gavage once per day for 16 wk.

At 24 wk of age, following a 12-h fast, blood samples were collected from the ophthalmic vein of mice under anesthesia. Systolic arterial blood pressure (SABP) was measured by tail-cuff micro-photoelectric plethysmography. Fasting blood was collected from the mice. Fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c), total cholesterol (TC), triglyceride (TG), lactate dehydrogenase (LDH), creatine kinase isoenzyme (CK-MB), troponin (cTnI), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured using the automatic biochemical instrument of the Second Hospital of Hebei Medical University. Cardiac function was evaluated by echocardiography. The mice were killed by cervical dislocation after 4% chloral hydrate anesthesia (0.20 mL/20 g; i.p.), and the cardiac muscle tissues were collected during chest surgery for analysis.

Cardiac echocardiography

Mice were anesthetized using 1.5% maintenance of isoflurane, and their heart rates were maintained between 400 and 500 beats/min. VEVO 3100 imaging system (Visual Sonics Inc., Toronto, ON, Canada) was used to perform echocardiography under M-mode. The left ventricle, the left ventricular ejection fraction (LVEF), the left ventricular fractional shortening (LVFS), the left ventricular internal dimension in systole (LVIDs), and the left ventricular end-diastolic diameter (LVIDd) were recorded.

Hematoxylin and eosin and Masson staining

The heart tissues were fixed in 4% paraformaldehyde and then embedded in paraffin. Tissue sections from the paraffin-embedded samples were stained following hematoxylin and eosin (HE) and Masson's trichrome staining. The myocardial staining was visualized and recorded under an optical microscope. HE and Masson staining was used to examine the changes of morphology and collagen fibers in myocardial tissues. Three sections and fields were investigated in histological evaluations in each group.

Immunohistochemistry and immunofluorescence staining

Specimens of myocardium were fixed in 4% paraformaldehyde and embedded in paraffin. The paraffin blocks were cut into 5-μm thick sections and heated for 10 min in 0.01 mol/L sodium citrate buffer with a microwave oven for antigen retrieval. Subsequently, 3% hydrogen peroxide was added to quench endogenous peroxidase activity. The sections were then blocked with 10% nonimmune goat serum to reduce nonspecific binding and incubated with 1:200 diluted anti-TGF-β1, 1:500 diluted anti-TNF-α, and 1:500 diluted anti-IL-1β antibodies (Abways, China) overnight at 4°C. After washing in phosphate-buffered saline (PBS), horseradish peroxidase (HRP)-conjugated secondary antibody was added and incubated with diaminobenzidine tetrahydrochloride. The sections were mounted on slides, stained with hematoxylin, and dehydrated in graded alcohol.

The paraffin blocks were cut into 5-μm thick sections, blocked with 10% nonimmune goat serum to reduce nonspecific binding, incubated with 1:100 diluted CD206 (Santa Cruz Biotechnology, Dallas, TX, USA) and 1:100 diluted inducible nitric oxide synthase (iNOS) (eBioscience, San Diego, CA, USA) antibodies overnight at 4C. After washing in PBS, HRP-conjugated secondary antibody was added and incubated for an additional 30 min at 37C. 4,6-diamidino-2-phenylindole (DAPI) was used to detect the nucleated cells. Images were visualized under a fluorescence microscope (Olympus, Tokyo, Japan). Three sections and fields were investigated for histological evaluations in each group.

ELISA

The levels of TGF-β1, IL-1β, and TNF-α in the serum samples were detected using ELISA kits (Multi Sciences, Hangzhou, China). Fasting insulin (FINS) was measured using another ELISA kit (Senberga, Nanjing, China).

Measurement of malondialdehyde and superoxide dismutase

Cardiac muscle tissues were homogenized, and the supernatants were harvested. Malondialdehyde (MDA) concentrations and superoxide dismutase (SOD) activities in myocardium were examined using commercial assay kits (Nanjing Jiancheng Biological Engineering Institute, Nanjing, China).

Real-time quantitative polymerase chain reaction

Total RNA was extracted using RNA-easyTM Isolation Reagent (Vazyme, Nanjing, China), and real-time quantitative polymerase chain reaction (RT-qPCR) was performed using GoTaq qPCR Master Mix (Promega, Madison, WI, USA). The primers were provided by Sangon Biotechnology (Shanghai, China). The primers are shown in Table 1 The relative expression of the target mRNA was calculated by the $2^{\Delta\Delta CT}$ method.

Statistical analysis

Data were analyzed by GraphPad Prism.9.0 software (GraphPad, La Jolla, CA, USA) and expressed as mean ± SD. The differences among multiple groups were analyzed using one-way analysis of variance, followed by the Tukey test if F was significant. $P < 0.05$ was considered a statistical difference.

RESULTS

Effects of atorvastatin on biochemical parameters and SABP in db/db mice

FBG, FINS, HbA1c, TG and TC of the db/db mice were higher than those of the control mice (Table 2). Atorvastatin treatment of db/db mice markedly decreased their TC, but had no effects on FBG, FINS, HbA1c or TG. The db/db mice treated with metformin exhibited lower levels of FBG, FINS and HbA1c than those in the atorvastatin treatment group. There were no significant differences in SABP among the four groups.

Effects of atorvastatin on cardiac function and structure in db/db mice

db/db mice had significant increased LVIDd and LVIDs, accompanied by a significant reduction in LVFS and LVEF, while the atorvastatin treatment significantly decreased their LVIDd level and augmented their LVFS and LVFF levels (Figure 1). Metformin treatment of db/db mice decreased LVIDd and LVIDs, but had no effects on LVFS and LVEF. The heart weight/body weight (HW/BW) of the db/db mice was significantly higher than that of the control mice, and treatment with atorvastatin or metformin significantly lowered HW/BW (Table 2). To evaluate the histological changes in the myocardium, HE and Masson staining was performed. db/db mice displayed disorganized and broken myocardial fibers and irregular nucleus and deposition of collagen in the myocardial interstitium, which were relieved by either atorvastatin or metformin treatment, especially atorvastatin (Figure 2). Compared with the control mice, the db/db mice had elevated serum levels of CK-MB, LDH and cTnI, indicating myocardial injury, while atorvastatin or metformin treatment decreased the serum levels of LDH, CK-MB and cTnI in db/db mice (Figure 3).

Effects of atorvastatin on inflammation and oxidative stress in db/db mice

Compared with the control mice, the serum levels and mRNA expression of TGF-β1, TNF-α and IL-1β in the myocardium were markedly elevated, while atorvastatin treatment markedly lowered these indicators in the serum and myocardium of db/db mice. Metformin treatment of db/db mice had no effects on the mRNA expression of IL-1β in the myocardium. The results of immunohistochemical staining of TGF-β1, TNF-α and IL-1β in the myocardium were consistent with those in the serum (Figures 3 and 4). Compared with the control mice, MDA concentration in cardiac muscle tissues of db/db mice was significantly increased, while SOD activity was significantly decreased. Compared with the db/db mice, atorvastatin or metformin treatment reduced MDA concentration and enhanced SOD activity in the myocardium (Figure 5).

Effects of atorvastatin on myocardial macrophage phenotypes in db/db mice

Immunofluorescence staining of macrophages showed increased M1 proinflammatory macrophages ($INOS^+$) and decreased M2 anti-inflammatory macrophages ($CD206^+$) in the myocardium of db/db mice. Atorvastatin treatment reduced the expression of M1 macrophages and promoted expression of M2 macrophages. However, metformin treatment increased expression of M2 macrophages and had no effects on the expression of M1 macrophages in the myocardium of db/db mice (Figure 5).

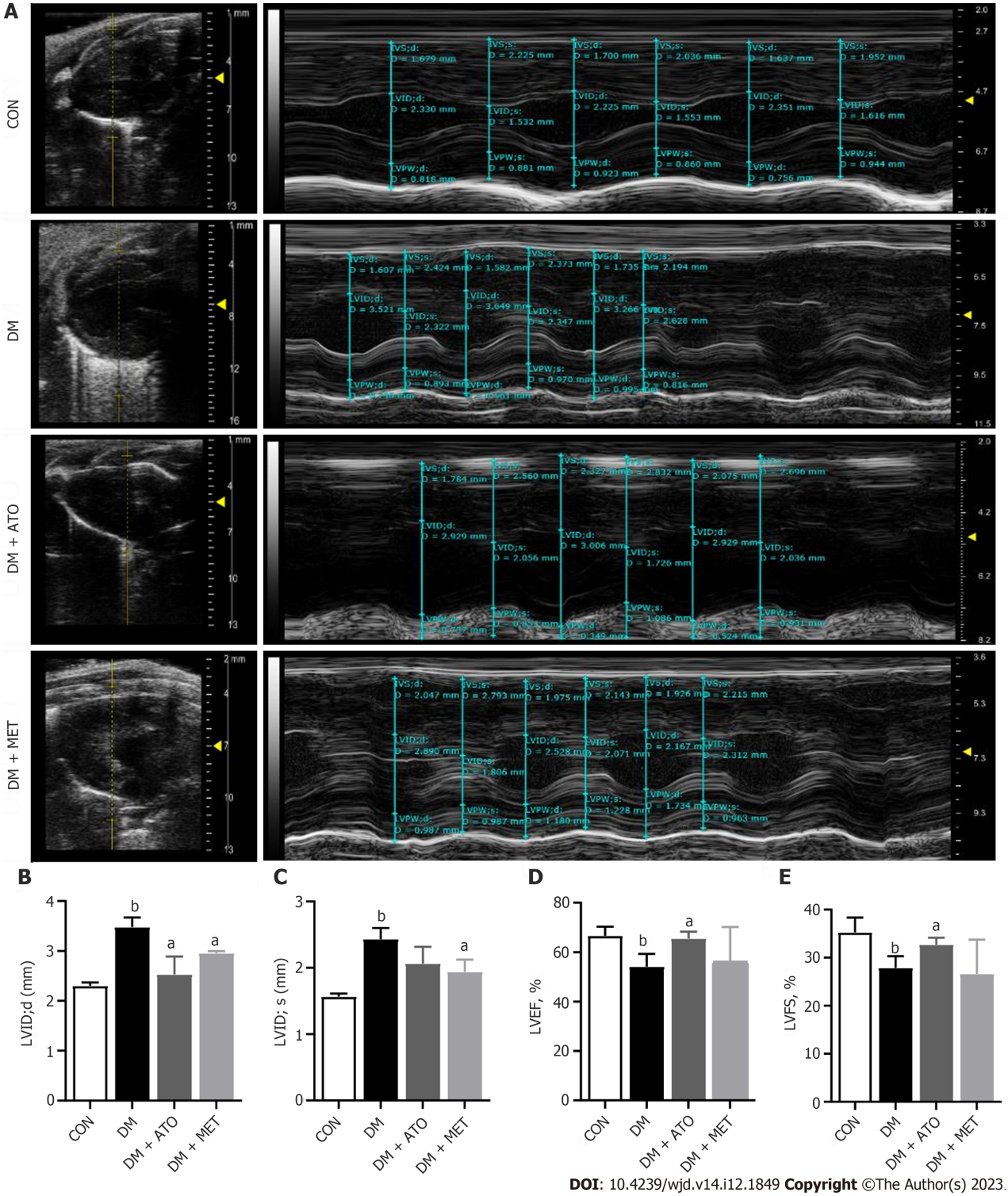

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Figure 1 Comparison of echocardiographic indices of cardiac systolic and diastolic function between each group. A: Representative pictures of cardiac echocardiography in each group; B–E: db/db mice received daily oral gavage of sterilized water (DM group) showed a significant increase in left ventricular end-diastolic diameter (LVIDd) and left ventricular internal dimension in systole (LVIDs), accompanied by a significant reduction in left ventricular fractional shortening (LVFS) and left ventricular ejection fraction (LVEF), while db/db mice that received daily oral gavage of 10 mg/kg/d atorvastatin group showed decreased levels of LVIDd, and augmented levels of LVFS and LVEF. The DM+MET group had decreased levels of LVIDd and LVIDs, but no effects on LVFS and LVEF. Data represent the means ± SD (n = 5). ^{a}P < 0.05 compared with db/db mice received daily oral gavage of sterilized water group. ^{b}P < 0.05 compared with C57BL/6 mice. DM: db/db mice received daily oral gavage of sterilized water; DM + ATO: db/db mice received daily oral gavage of 10 mg/kg/d atorvastatin; DM + MET: db/db mice received daily oral gavage of 200 mg/kg metformin; CON: C57BL/6 mice; LVEF: Left ventricular ejection fraction; LVFS: Left ventricular fractional shortening; LVIDs: Left ventricular internal dimension in systole; LVIDd: Left ventricular end-diastolic diameter.

Table 1 Primers used in this study

Gene	Primer	Tm (°C)	Product length	Accession
TGF-β1	Forward: 5′-CTCCCGTGGCTTCTAGTGC-3′	60.15	133	NM_011577.2
	Reverse: 5′-GCCTTAGTTTGGACAGGATCTG-3′	58.73		
TNF-α	Forward: 5′-CCCTCACACTCAGATCATCTTCT-3′	59.29	61	NM_013693.3
	Reverse: 5′-GCTACGACGTGGGCTACAG-3′	60.23		
IL-1β	Forward: 5′-GCAACTGTTCCTGAACTCAACT-3′	59.05	89	NM_008361.4
	Reverse: 5′-ATCTTTTGGGGTCCGTCAACT-3′	59.58		
18S rRNA	Forward: 5′-AGGGGAGAGCGGGTAAGAGA-3′	61.58	241	AH002077.2
	Reverse: 5′-GGACAGGACTAGGCGGAACA-3′	61.26		

Tm: Melting temperature; TGF: Transforming growth factor; TNF: Tumor necrosis factor; IL: Interleukin.

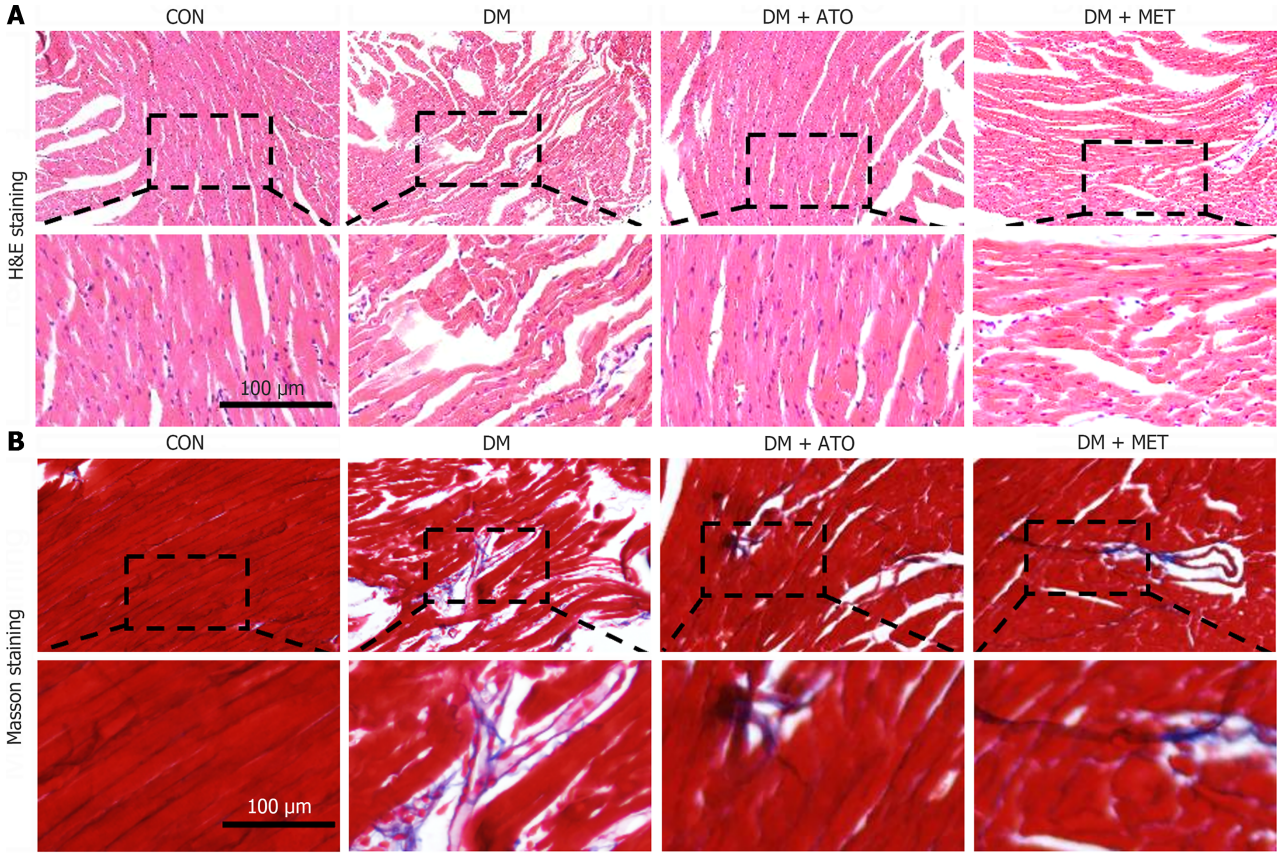

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Figure 2 Histological changes in the myocardium. A: Hematoxylin and eosin staining was performed for each group. db/db mice that received daily oral gavage of sterilized water (DM group) had disorganized and broken myocardial fibers and irregular nuclei, which were relieved in db/db mice by daily oral gavage of 10 mg/kg/d atorvastatin (DM + ATO) or in db/db mice by daily oral gavage of 200 mg/kg metformin (DM + MET) group, especially in the DM + ATO group; B: Masson staining was performed for each group. DM group displayed deposition of collagen in the myocardial interstitium, which was relieved in the DM + ATO or DM + MET group, especially the DM + ATO group. Scale bar, 100 μm. DM: db/db mice received daily oral gavage of sterilized water; DM + ATO: db/db mice received daily oral gavage of 10 mg/kg/d atorvastatin; DM + MET: db/db mice received daily oral gavage of 200 mg/kg metformin; CON: C57BL/6 mice; H&E: Hematoxylin and eosin staining.

DISCUSSION

The incidence of HF has increased in patients with DM, which is closely related to DCM. The development of DCM initiates from subtle myocardial changes to myocardial fibrosis and diastolic dysfunction and eventually to stubborn HF. Cardiac fibrosis is one of the primary characteristics of DCM that contributes to the development of adverse cardiac remodeling and myocardial stiffness[24]. Hyperglycemia, hyperlipidemia, hyperinsulinemia, and impaired insulin

Table 2 Biochemical parameters, systolic arterial blood pressure and ratio of heart weight/body weight in mice at 24-wk age

Parameters	CON, *n* = 5	DM, *n* = 5	DM + ATO, *n* = 5	DM + MET, *n* = 5
FBG (mmol/L)	5.12 ± 0.11	31.81 ± 3.27[b]	33.19 ± 3.13[b]	10.16 ± 2.92[a,c,d]
FINS (mU/L)	62.82 ± 4.98	281.24 ± 15.47[b]	290.10 ± 18.39[b]	104.13 ± 8.99[a,c,d]
HbA1c (%)	5.51 ± 0.60	9.57 ± 0.58[b]	9.42 ± 0.47[b]	7.15 ± 0.84[a,c,d]
ALT (IU/L)	58.95 ± 9.88	67.75 ± 11.30	69.03 ± 14.29	73.49 ± 15.68
AST (IU/L)	131.3 ± 27.12	157.3 ± 30.36	139.7 ± 28.49	143.9 ± 32.19
TG (mmol/L)	1.10 ± 0.17	2.48 ± 0.46[b]	2.29 ± 0.33[b]	2.17 ± 0.25[b]
TC (mmol/L)	2.22 ± 0.25	3.77 ± 0.39[a]	2.15 ± 0.38[c]	3.29 ± 0.32[a,d]
SABP (mmHg)	123.9 ± 3.09	133.9 ± 5.96	129.6 ± 4.88	136.4 ± 5.71
HW/BW (× 10^{-3})	4.37 ± 0.55	6.25 ± 0.38[a]	4.89 ± 0.10[c]	5.01 ± 0.46[c]

[a]$P < 0.05$ *vs* control group.
[b]$P < 0.01$ *vs* control group.
[c]$P < 0.05$ *vs* db/db mice group.
[d]$P < 0.05$ *vs* db/db mice + atorvastatin.
CON: Control group; DM: db/db mice group; DM+ATO: db/db mice + atorvastatin; DM+MET: db/db mice + metformin; FBG: Fasting blood glucose; FINS: Fasting insulin; HbA1c: Hemoglobin A1c; ALT: Alanine transaminase; AST: Aspartate aminotransferase; TG: Triglyceride; TC: Total cholesterol; HW/BW: Heart weight/body weight; SABP: Systolic arterial blood pressure.

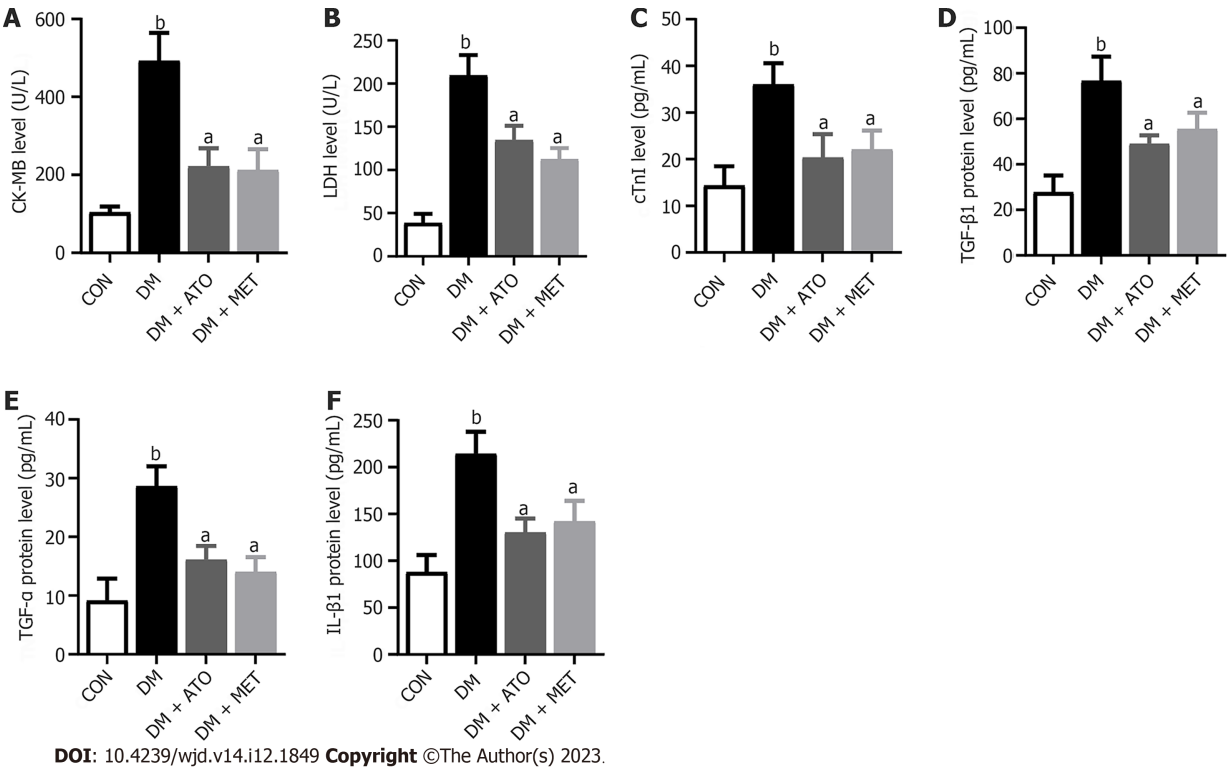

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Figure 3 Expression of markers of cardiac injury and indicators of inflammation in the serum of each group. A–C: Serum creatine kinase isoenzyme (CK-MB), lactate dehydrogenase (LDH) and troponin (cTnI) were measured using the automatic biochemical instrument. Compared with C57BL/6 mice (CON group), db/db mice that received daily oral gavage of sterilized water (DM group) had elevated serum CK-MB, LDH and cTnI. db/db mice that received daily oral gavage of 10 mg/kg/d atorvastatin (DM + ATO) or db/db mice that received daily oral gavage of 200 mg/kg metformin (DM + MET) group had decreased serum CK-MB, LDH and cTnI; D–F: The levels of transforming growth factor (TGF)-β1, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β in the serum samples were detected by ELISA. Compared with CON group, the serum levels of TGF-β1, TNF-α, and IL-1β were markedly elevated. In the DM + ATO and DM + MET groups, these indicators were markedly lower. Data represent the means ± SD ($n = 5$). [a]$P < 0.05$ compared with db/db mice received daily oral gavage of sterilized water group. [b]$P < 0.05$ compared with C57BL/6 mice. DM: db/db mice received daily oral gavage of sterilized water; DM + ATO: db/db mice received daily oral gavage of 10 mg/kg/d atorvastatin; DM + MET: db/db mice received daily oral gavage of 200 mg/kg metformin. CON: C57BL/6 mice.

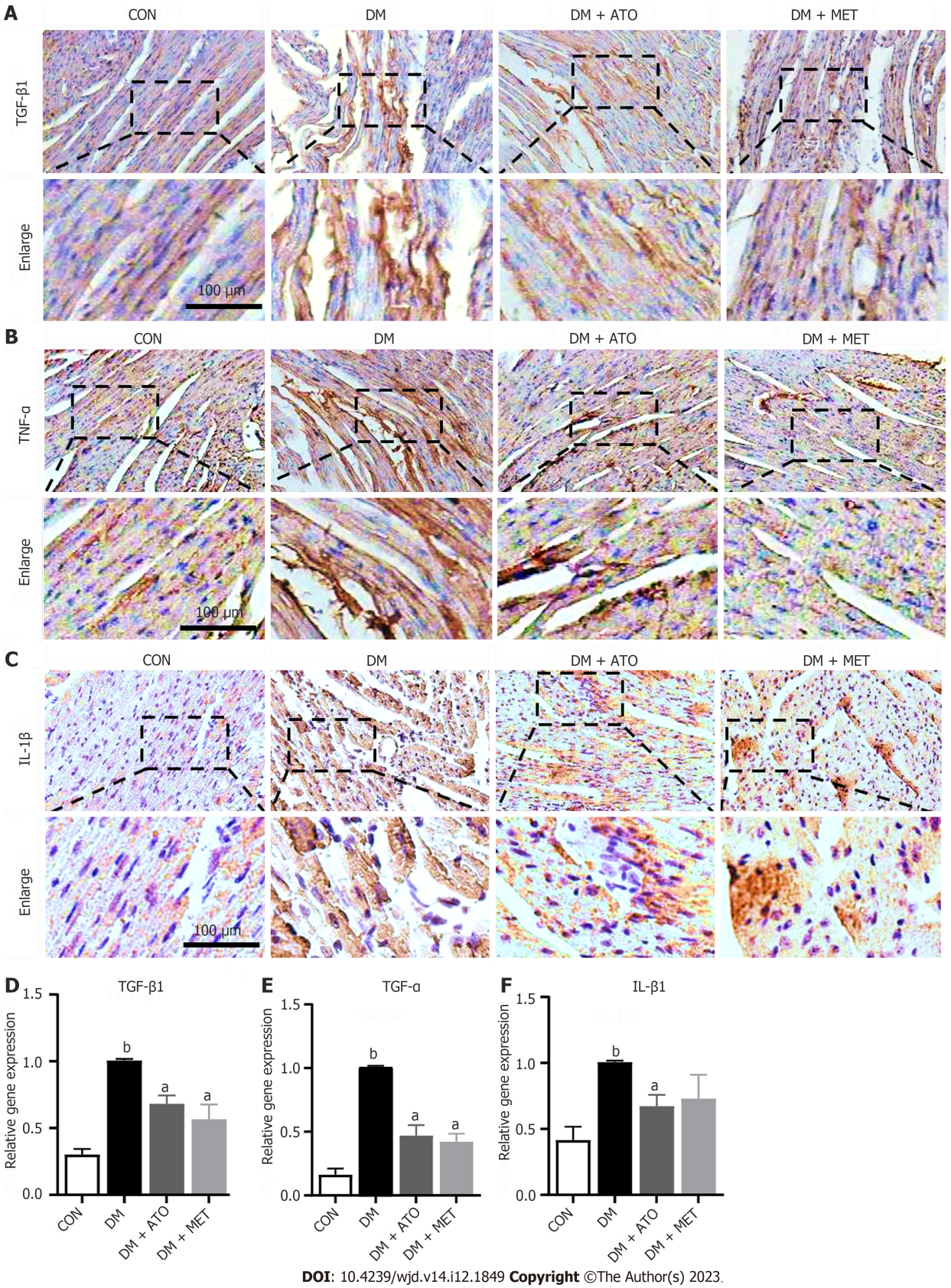

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Figure 4 Immunohistochemical staining and relative gene expression of transforming growth factor-β1, tumor necrosis factor-α, and interleukin-1β in each group. A–C: Immunohistochemistry staining of TGF-β1, TNF-α, and IL-1β in each group; D–F: relative gene expression of TGF-β1, TNF-α, and IL-1β in each group. Compared with C57BL/6 mice (CON group), the expression of TGF-β1, TNF-α and IL-1β in db/db mice that received daily oral gavage of sterilized water (DM) group was markedly elevated, and daily oral gavage of 10 mg/kg/d atorvastatin (DM + ATO) or 200 mg/kg metformin (DM + MET) alleviated these manifestations. The DM + MET group showed no effects on IL-1β mRNA expression in the myocardium. Data represent the means ± SD (n = 5). $^{a}P < 0.05$ compared with db/db mice received daily oral gavage of sterilized water group. $^{b}P < 0.05$ compared with C57BL/6 mice. DM: db/db mice received daily oral gavage of

sterilized water; DM+ATO: db/db mice received daily oral gavage of 10 mg/kg/d atorvastatin; DM+MET: db/db mice received daily oral gavage of 200 mg/kg metformin; CON: C57BL/6 mice; TGF-β1: Transforming growth factor-β1; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β.

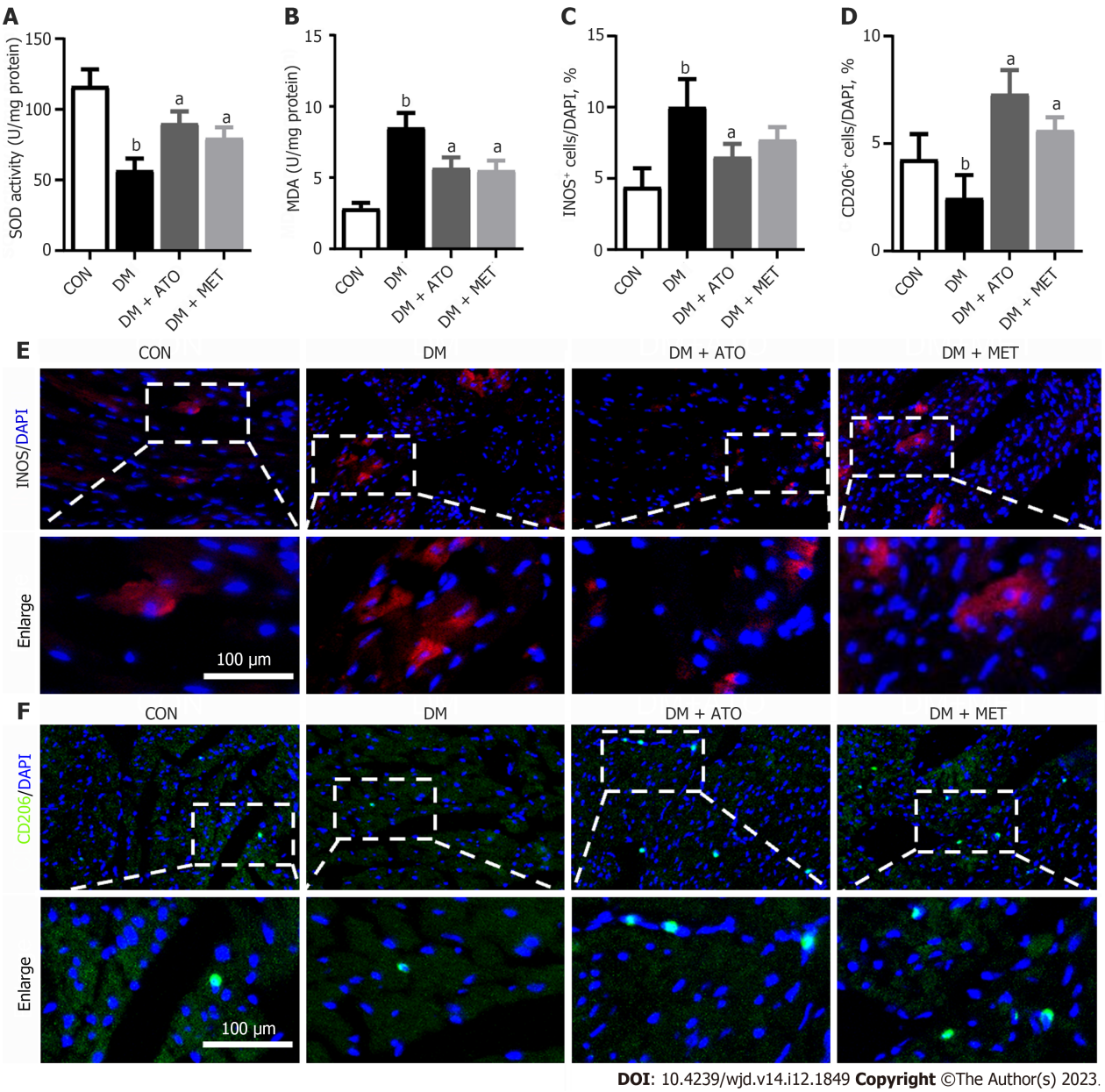

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Figure 5 Levels of superoxide dismutase activity and malondialdehyde content and immunofluorescence staining of macrophages. A and B: SOD activity was decreased and MDA content was significantly increased in db/db mice that received daily oral gavage of sterilized water (DM group). db/db mice that received daily oral gavage of 10 mg/kg/d atorvastatin (DM + ATO) or 200 mg/kg metformin (DM + MET) group had markedly decreased MDA content and enhanced SOD activity; C and E: Compared with C57BL/6 mice (CON group), iNOS$^+$ in the DM group were markedly increased, and the DM + ATO group had reduced the expression of M1 macrophages. The DM + MET group showed no effects on expression of M1 macrophages in the myocardium of db/db mice; D and F: Compared with the CON group, CD206$^+$ in the DM group were markedly decreased, and the DM + ATO and DM + MET groups had increased expression of M2 macrophages in the myocardium. Data represent the means ± SD (n = 5). $^a P < 0.05$ compared with db/db mice received daily oral gavage of sterilized water group. $^b P < 0.05$ compared with C57BL/6 mice. iNOS: Inducible nitric oxide synthase; MDA: Malondialdehyde; SOD: Superoxide dismutase; DM: db/db mice received daily oral gavage of sterilized water; DM + ATO: db/db mice received daily oral gavage of 10 mg/kg/d atorvastatin; DM + MET: db/db mice received daily oral gavage of 200 mg/kg metformin; CON: C57BL/6 mice.

signaling are the main initiators of DCM[25]. db/db mice are often used as a typical animal model of T2DM. In our study, the db/db mice exhibited hyperglycemia, hyperlipidemia and hyperinsulinemia, indicating a feature of T2DM. At 24 wk old, the db/db mice showed a significant increase in LVIDd and LVIDs and a significant reduction in LVFS and LVEF, as shown by echocardiography, suggesting cardiac diastolic and systolic dysfunction. The HW/BW of the db/db mice was significantly higher than that of the control mice; in combination with the results from HE and Masson staining, these findings manifested as myocardial pathological hypertrophy and fibrosis in the db/db mice. The elevated serum levels of

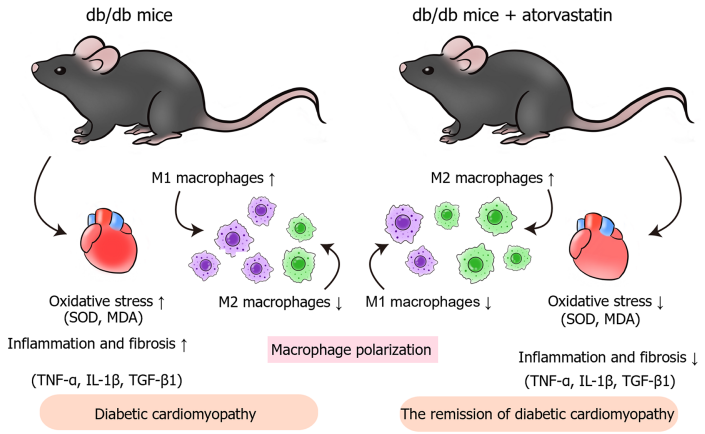

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Figure 6 Graphical abstract. MDA: Malondialdehyde; SOD: Superoxide dismutase; TGF-β1: Transforming growth factor-β1; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β.

CK-MB, LDH and cTnI also suggested myocardial injury of the db/db mice. Therefore, the db/db mice had developed DCM.

Metformin is a widely used glucose-lowering drug and has been confirmed to exert cardioprotective effects by improving the morphology and structure of the heart in db/db mice[20,26]. Metformin ameliorates DCM by inhibiting myocardial inflammation and oxidative stress, mainly through the activation of mitogen-activated protein kinase and promotion of autophagic flux[20,27,28]. At present, there is ample clinical evidence supporting the notion that metformin can reduce the risk of cardiovascular rehospitalization in diabetic patients with HF, and decrease the high risk of exacerbating DCM in prediabetic patients[29,30]. A recent meta-regression analysis has demonstrated that metformin is associated with reduced mortality in patients with HF with preserved ejection fraction, resulting in an 18% decrease in mortality for all HF patients[31]. Although metformin can be used safely in T2DM patients complicated with HF, it should be noted that currently metformin is drugs and have become the first-line choice for patients suffering from cardiovascular diseases. In addition to lowering blood fat, statins possess multiple pleiotropic effects. Previous studies have shown that statins can prevent DCM by alleviating myocardial fibrosis through antioxidative stress and anti-inflammatory pathways[33,34]. Greig *et al*[35] showed that atorvastatin reduced the levels of oxidative stress and inflammation and restored endothelial dysfunction in patients with HF. Taking a daily dose of 40 mg of simvastatin reduced the risk of major adverse cardiovascular events by ~25% in diabetic patients[36]. Similarly, our study also demonstrated that atorvastatin inhibited myocardial injury and fibrosis, which contribute to DCM attenuation.

The occurrence and development of DCM is accompanied by oxidative stress and chronic inflammation[37,38]. Oxidative stress can prompt the transformation of fibroblasts to myofibroblasts, leading to cardiac fibrosis[39,40]. The present study displayed that atorvastatin had antioxidant properties in diabetic hearts. Cardiac tissues include a plenty of resident macrophages. Under high glucose conditions, macrophages can upregulate glucose uptake and utilization and enhance the production of inflammatory cytokines, such as TGF-β1 and TNF-α, which act on macrophages and promote the activation of inflammatory phenotype M1[41]. Dysregulation of macrophages between M1 and M2 phenotypes causes excessive inflammation and cardiac injury[42]. Macrophages can also promote cardiac fibrosis through either directly producing extracellular matrix proteins or stimulating fibroblasts to secret TGF-β1[25]. Widiapradja *et al*[6] showed that only M2 macrophages were found in normal mouse hearts without inflammation, but there was a predominant increase in M1 macrophages in diabetic hearts, leading to a significant increased M1/M2 ratio. Liu *et al*[43] also had similar findings for the hearts of T2DM mice. We observed the distribution of M1 ($CD86^+$) and M2 ($CD206^+$) macrophages in the hearts of the db/db mice and found that M1 macrophages were increased in diabetic hearts. It has been reported that the imbalance of M1/M2 ratio can accelerate the development of DCM[17], and the regulation of macrophage polarization can improve the cardiac function of DCM[18]. These results demonstrate that inflammatory polarization of macrophages plays an important role in the development of DCM. Our study showed that atorvastatin reduced the expression of M1 macrophages and increased M2 macrophages in the hearts of db/db mice, and concurrently decreased the levels of TGF-β 1, TNF-α and IL-1β in both the myocardium and the serum. These findings are consistent with those from the study by Jia *et al*[28].

CONCLUSION

Our study suggests that administration of atorvastatin attenuates myocardial fibrosis in db/db mice, which may be associated with the antioxidative stress and anti-inflammatory effects of atorvastatin on diabetic myocardium through modulating macrophage polarization. The investigation of cardiac macrophage polarization will facilitate DCM treatment by targeting macrophage metabolism in the hearts (Figure 6).

ARTICLE HIGHLIGHTS

Research background

Statins were initially used to lower blood lipids; however, in addition to their lipid-lowering effects, statins are involved in the regulation of the inflammatory response and play an important role in cardiovascular protection. Macrophage polarization is involved in a variety of pathological processes. Macrophage polarization is likewise involved in the development of diabetic cardiomyopathy (DCM).

Research motivation

DCM is one of the serious complications of diabetes mellitus, and we wanted to explore whether atorvastatin could mitigate the effects on DCM by affecting macrophage polarization to reduce oxidative stress, inflammation, and cardiac fibrosis.

Research objectives

We used db/db mice as a type 2 diabetes model and randomly divided into three groups: The db/db mice received daily oral gavage of sterilized water group, atorvastatin group and metformin group. C56BL/6 mice were used as the control group.

Research methods

Cardiac function was evaluated by echocardiography. Histological evaluations are hematoxylin and eosin staining, Masson staining, immunohistochemistry, and immunofluorescence. ELISA and real-time quantitative polymerase chain reaction were also used.

Research results

Treatment with atorvastatin improved cardiac dysfunction in db/db mice. Atorvastatin reduced the levels of serum myocardial injury markers; lowered the levels of Inflammatory cytokines in serum and myocardium; decreased indicators of oxidative stress in myocardium of db/db mice; inhibited M1 macrophages and promoted M2 macrophages.

Research conclusions

Administration of atorvastatin attenuates myocardial fibrosis in db/db mice, which may be associated through modulating macrophage polarization.

Research perspectives

Our study further confirms the protective role of statins in cardiovascular disease and provides a new therapeutic target for DCM.

FOOTNOTES

Author contributions: Zhou H designed the study and revised the manuscript; Song XM performed the experiments and drafted the manuscript; Zhao MN participated in data processing and revised the manuscript; Li GZ and Li N contributed to animal feeding; Wang T performed statistical analyses; and all authors contributed to the article and approved the submission of this manuscript.

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Data sharing statement: Data will be made available on request.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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ORIGINAL ARTICLE

Basic Study

Empagliflozin ameliorates diabetic cardiomyopathy probably *via* activating AMPK/PGC-1α and inhibiting the RhoA/ROCK pathway

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Abstract

BACKGROUND

Diabetic cardiomyopathy (DCM) increases the risk of hospitalization for heart failure (HF) and mortality in patients with diabetes mellitus. However, no specific therapy to delay the progression of DCM has been identified. Mitochondrial dysfunction, oxidative stress, inflammation, and calcium handling imbalance play a crucial role in the pathological processes of DCM, ultimately leading to cardiomyocyte apoptosis and cardiac dysfunctions. Empagliflozin, a novel glucose-lowering agent, has been confirmed to reduce the risk of hospitalization for HF in diabetic patients. Nevertheless, the molecular mechanisms by which this agent provides cardioprotection remain unclear.

AIM

To investigate the effects of empagliflozin on high glucose (HG)-induced oxidative stress and cardiomyocyte apoptosis and the underlying molecular mechanism.

METHODS

Twelve-week-old db/db mice and primary cardiomyocytes from neonatal rats stimulated with HG (30 mmol/L) were separately employed as *in vivo* and *in vitro* models. Echocardiography was used to evaluate cardiac function. Flow cytometry and TdT-mediated dUTP-biotin nick end labeling staining were used to assess apoptosis in myocardial cells. Mitochondrial function was assessed by cellular ATP levels and changes in mitochondrial membrane potential. Furthermore, intracellular reactive oxygen species production and superoxide dismutase activity were analyzed. Real-time quantitative PCR was used to analyze *Bax* and *Bcl-2* mRNA expression. Western blot analysis was used to measure the phosphorylation of AMP-activated protein kinase (AMPK) and myosin phosphatase target subunit 1 (MYPT1), as well as the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and active caspase-3 protein levels.

RESULTS

In the *in vivo* experiment, db/db mice developed DCM. However, the treatment of db/db mice with empagliflozin (10 mg/kg/d) for 8 wk substantially enhanced cardiac function and significantly reduced myocardial apoptosis, accompanied by an increase in the phosphorylation of AMPK and PGC-1α protein levels, as well as a decrease in the phosphorylation of MYPT1 in the heart. In the *in vitro* experiment, the findings indicate that treatment of cardiomyocytes with empagliflozin (10 μM) or fasudil (FA) (a ROCK inhibitor, 100 μM) or overexpression of PGC-1 α significantly attenuated HG-induced mitochondrial injury, oxidative stress, and cardiomyocyte apoptosis. However, the above effects were partly reversed by the addition of compound C (CC). In cells exposed to HG, empagliflozin treatment increased the protein levels of p-AMPK and PGC-1α protein while decreasing phosphorylated MYPT1 levels, and these changes were mitigated by the addition of CC. Adding FA and overexpressing PGC-1α in cells exposed to HG substantially increased PGC-1α protein levels. In addition, no sodium-glucose cotransporter (SGLT)2 protein expression was detected in cardiomyocytes.

CONCLUSION

Empagliflozin partially achieves anti-oxidative stress and anti-apoptotic effects on cardiomyocytes under HG conditions by activating AMPK/PGC-1α and suppressing of the RhoA/ROCK pathway independent of SGLT2.

Key Words: Empagliflozin; Diabetic cardiomyopathy; AMPK; ROCK; Apoptosis; Oxidative stress

Core Tip: We established a diabetic cardiomyopathy model in db/db mice and treated the mice with empagliflozin for 8 wk, and found that empagliflozin observably improved cardiac function in diabetic mice, which was maybe related to activation of AMP-activated protein kinase (AMPK)/peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and inhibition of the RhoA/ROCK pathway. In order to exclude the effects of metabolic improvement on the heart *in vivo*, *in vitro* experiment in high glucose conditions was performed. The results confirmed that the anti-oxidative stress and anti-apoptotic effects of empagliflozin on cardiomyocytes were achieved by activating AMPK/PGC-1α and inhibiting ROCK. Furthermore, the effects were independent of sodium-glucose cotransporter (SGLT)2 inhibition as no SGLT2 expression was detected on cardiomyocytes.

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INTRODUCTION

Diabetic cardiomyopathy (DCM) is characterized by systolic and diastolic dysfunction, eventually resulting in heart failure (HF) in patients with diabetes mellitus (DM) in the absence of hypertension, coronary artery disease, and valvular heart disease[1,2]. To date, no specific treatments have been identified to delay the progression of DCM. The pathological processes of DCM include insulin resistance, mitochondrial dysfunction, oxidative stress, calcium handling imbalance, and inflammation[3]. In hyperglycemia environments, reduced antioxidant enzymes and increased production of reactive oxygen species (ROS), defined as oxidative stress[4], affect various signaling pathways involved in the occurrence of DCM, ultimately leading to cardiomyocyte apoptosis and cardiac dysfunction[5]. Myocardial oxidative stress and apoptosis are key components of its pathogenesis, and their occurrence has been confirmed in patients with DM and diabetic animal models[6,7].

Sodium-glucose cotransporter (SGLT)2 inhibitors are a novel class of glucose-lowering agents that enhance urinary glucose excretion combined with excessive natriuresis independently of insulin. Clinical trials have demonstrated that SGLT2 inhibitors substantially reduced the risk of hospitalization for HF in patients with DM[8-10]. In addition, the cardiac benefits of empagliflozin have been demonstrated in non-diabetic patients with HF and reduced ejection fraction [11]. However, the mechanism by which these observed benefits are mediated remains unclear. Two systematic reviews and meta-analyses demonstrated that the reversal of cardiac remodeling might be a mechanism responsible for the favorable clinical effects of these agents on HF regardless of glycemic status, particularly in the case of empagliflozin[12, 13]. Adverse cardiac remodeling is closely associated with increased myocardial apoptosis, decreased autophagy, and altered energy metabolism in the heart[14]. Packer speculated that the cardioprotective effects of SGLT2 inhibitors might be due to the direct effects of these drugs on cardiomyocytes, involving the activation of AMP-activated protein kinase (AMPK), reduction of cellular stress, and restoration of cellular survival[15]. However, further experiments are required to validate the molecular mechanisms underlying the benefits of SGLT2 inhibitors on the heart.

AMPK, which is activated by an increased AMP/ATP ratio, plays a crucial role in regulating the energy metabolism of the heart[16]. Recent investigations have demonstrated that empagliflozin protects the heart from inflammation and energy depletion, and it improves myocardial vascular injury in diabetic mice through the activation of AMPK-mediated inhibition of mitochondrial fission and oxidative stress[17,18]. In addition, a recent study indicated that AMPK activation reduced the myocardial apoptotic effects in diabetic mice[19]. However, there is a scarcity of studies on SGLT2 inhibitors and cardiomyocyte apoptosis in DCM. Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) serves as a pivotal factor in maintaining mitochondrial biogenesis and energy metabolism and plays a critical role in the progression of HF. Deacetylation of silent mating type information regulation 2 homolog 1 (SIRT1) or phosphorylation of AMPK can modulate PGC-1α activity[20]. RhoA is a member of the GTP-binding proteins within the Ras superfamily, and Rho kinase (ROCK) is a serine/threonine protein kinase that acts as a principal effector of RhoA. Many important functions in cells, such as proliferation, apoptosis, and differentiation, and gene transcription are regulated by the RhoA/ROCK pathway. Our previous *in vitro* and *in vivo* studies have revealed that the RhoA/ROCK pathway regulates myocardial apoptosis and fibrosis in diabetic rats. Fasudil (FA), a ROCK inhibitor, has been shown to alleviate oxidative stress and improve cardiac function[21,22]. Therefore, the RhoA/ROCK pathway is associated with several cardiovascular conditions, such as hypertension, atherosclerosis, and HF[23].

In this study, we established a DCM model in db/db mice and then treated the mice with empagliflozin for 8 wk. Significant improvements in cardiac function were observed in diabetic mice, along with concurrent activation of AMPK/PGC-1α and the inhibition of the RhoA/ROCK pathway (Figures 1 and 2). An *in vitro* experiment under high glucose (HG) conditions was performed to exclude the effects of metabolic improvement on the heart *in vivo*. The aim of this study was to elucidate the molecular mechanisms underlying the protective effects of empagliflozin on cardiomyocytes.

MATERIALS AND METHODS

Animals

The experiments were conducted using 8-week-old male db/db mice weighing 40–42 g (Nanjing, China). The random blood glucose levels were ≥ 16.7 mmol/L in all db/db mice. Male C57BL/6J mice were used as a control group. The mice were given standard care according to a protocol approved by the Ethics Committee of Hebei Medical University. The animals were acclimatized to laboratory conditions (24 °C, 10 h/14 h light/dark, 55% humidity, *ad libitum* access to food and water) for 2 wk prior to experimentation. Intragastric gavage administration was carried out with conscious animals, using straight gavage needles appropriate for the animal size. All animals were euthanized by barbiturate overdose (intravenous injection, 200 mg/kg pentobarbital sodium) for tissue collection. All appropriate measures were taken to minimize the pain or discomfort of animals. Approval was granted by the Research Ethics Committee of the Second Hospital of Hebei Medical University (Date 2022.3.5/No. 2022-AE136). The mice were divided into three groups: (1) Normal control group (NG, n = 11); (2) Db/db mice group (DB, n = 7); and (3) empagliflozin (EM)-treated db/db mice group (EM/DB, n = 7). Empagliflozin (Biberach, Germany) was administrated *via* oral gavage at a dose of 10 mg/kg/d for 8 wk from the age of 12 wk.

Blood pressure, blood glucose, plasma insulin, and cholesterol determination

At the age of 20 wk, systolic arterial blood pressure (SABP) was measured by tail-cuff micro-photoelectric plethysmography. Fasting blood samples were collected for blood glucose (FBG), glycosylated hemoglobin (HbA1c), and total cholesterol (TC) determination using an automatic biochemical analyzer in the Second Hospital of Hebei Medical University (Shijiazhuang, China), and serum insulin (FINS) was measured using an ELISA kit (Senberga, Nanjing, China) according to the manufacturer's instructions. The insulin resistance index (HOMA-IR) was calculated as FBG × FINS/22.5.

Echocardiography

At the age of 20 wk, the mice were anesthetized with an intraperitoneal injection of pentobarbital sodium at a dose of 200 mg/kg. All measurements were performed with an 11-MHz linear transducer coupled to a high-resolution ultrasound system (Vivid E95, GE Healthcare, United States). Serial M-mode echocardiographic images were taken in the short axis view at the level of the papillary muscles. The left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), and peak velocity of early filling (E) and atrial contraction (A) were obtained to assess cardiac diastolic and systolic functions.

Transmission electron microscopy

The myocardial tissues were fixed with 2.5% glutaraldehyde overnight at 4 °C, followed by postfixation with 1% acetic acid. After dehydration, the specimens were conventionally processed and examined by transmission electron microscopy (TEM) (JEM-1200EX, JEOL, Japan) for analyses of the myocardium ultrastructure.

Histological staining

The heart tissues were fixed with 4% paraformaldehyde. The paraffin sections of heart tissues were dewaxed, stained with hematoxylin and eosin (HE), dehydrated, and mounted. A microscopic examination was performed to evaluate pathological changes in the heart tissues.

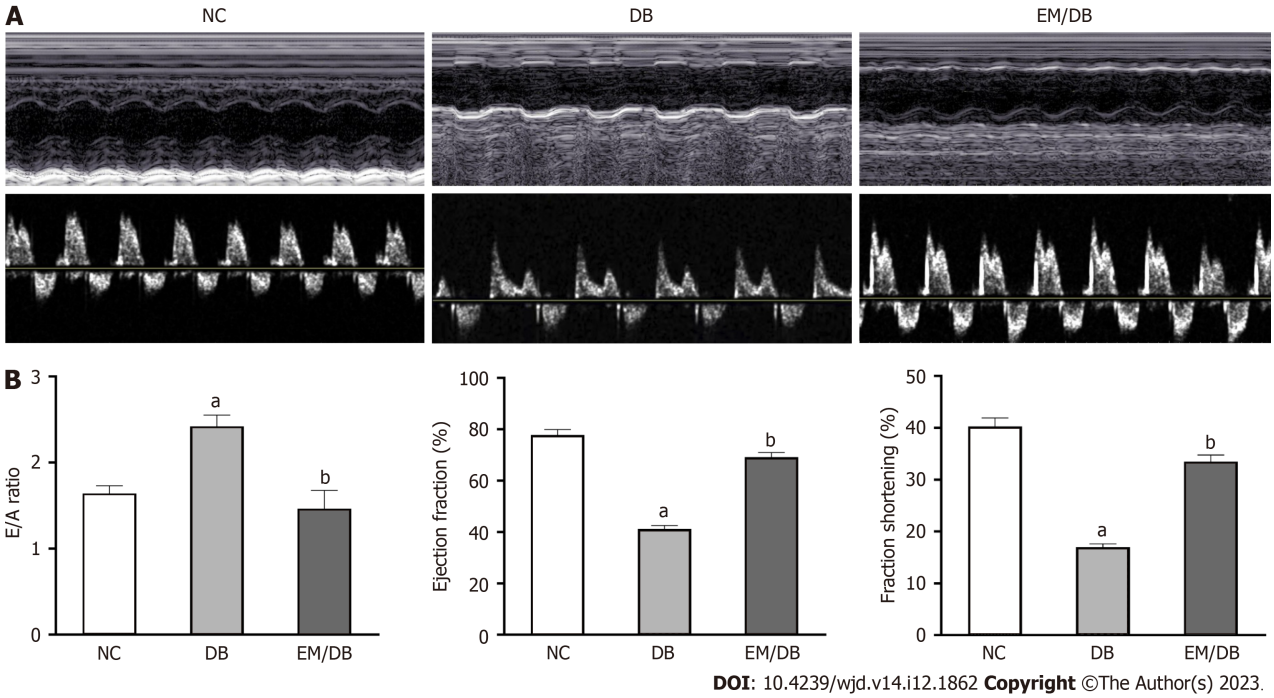

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Figure 1 Effects of empagliflozin on cardiac function in db/db mice. A: M-mode imaging revealed reduced systolic and diastolic function in db/db mice; B: Quantitation of E/A ratio, left ventricular ejection fraction, and left ventricular fractional shortening. NC: Normal control mice; DB: Db/db mice; EM/DB: Db/db mice treated with empagliflozin; LVEF: Left ventricular ejection fraction; LVFS: Left ventricular fractional shortening. $^{a}P < 0.05$ *vs* NC; $^{b}P < 0.05$ *vs* DB.

TdT-mediated dUTP-biotin nick end labeling assay of myocardial tissues

A TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay kit (Beyotime, Shanghai, China) was used to examine cell apoptosis in the myocardial tissues according to the manufacturer's instructions. Apoptotic cells were observed and photographed using a light microscope (CX2, OLYMPUS, Japan). The nuclei of apoptosis-positive cells were brown. The apoptotic index was calculated as the percentage of TUNEL-positive cells.

Cell culture and drug treatment

Cardiomyocytes were isolated from 1-3-day-old newborn Sprague-Dawley rats in the Animal Experimental Center of Hebei Medical University. Briefly, freshly isolated hearts were minced and washed thrice with D-Hanks (Gibco, Carlsbad, CA, United States), digested with a mixture of enzymes containing 0.04% type II collagenase (Biofroxx, Einhausen, Germany) and 0.1% trypsin (Gibco, Billings, MT, United States) for 8 min for 6 cycles, and then the digestion was stopped with fetal bovine serum (FBS; Gemini, CA, United States). Next, the tissues were disrupted in Dulbecco's Modified Eagle Medium (DMEM; Gibco, CA, United States) containing 10% FBS and 5.5 mmol/L D-glucose. Fibroblasts were eliminated by attaching to the culture plates for 90 min. Bromodeoxyuridine (BrdU, Solarbio, Beijing, China) was added to the medium to suppress the growth of fibroblasts, resulting in high-purity cardiomyocytes. These cells started spontaneously pulsating in about 3 d at 80-100 beats/min, and 95% of them, which were positive for anti-α-actin, were identified as cardiomyocytes by immunocytochemistry. This experiment was approved by the Experimental Animal Administration Committee of Hebei Medical University.

The isolated cardiomyocytes were maintained for 24 h in DMEM supplemented with streptomycin (100 μg/mL), penicillin (100 U/mL), and 5% FBS. Next, the cells were cultured in different conditions: 5.5 mmol/L D-glucose as normal control (NG) group; 5.5 mmol/L D-glucose plus 24.5 mmol/L D-mannitol as hyperosmotic group (OSM); 30 mmol/L D-glucose as HG group (HG); cells in the HG + EM group were pretreated with 10 μM empagliflozin for 2 h and then incubated with HG for an additional 48 h; cells in the HG + EM + compound C (CC) group were pretreated with AMPK inhibitor and 1 μM CC (MCE, CA, United States) for 2 h, and then the cells were cultured as in the HG + EM group; cells in the HG + FA group were pretreated with 100 μM FA (Hongri, Tianjin, China)) as our previous study[17] and incubated with HG for an additional 48 h; cells in the PGC-1α-GFP-Ad and GFP-Ad groups were transfected with PGC-1α-GFP-Ad and GFP-Ad (Shanghai Genechem Co., Ltd., China), respectively, using liposomes (Invitrogen, United States) and then incubated with HG for an additional 48 h.

Cell counting kit-8 measurement

Cell counting kit-8 (CCK-8) (Sharebio, Shanghai, China, SB-CCK8S) was used to measure cell viability. The cardiomyocytes were inoculated in the 96-well plate (100 μl/well), followed by intervention with empagliflozin for 48 h at different concentrations (0, 0.05, 0.1, 1, 10, and 20 μM) in HG conditions. The medium was then supplemented with CCK-8 solution for 4 h. Lastly, a microplate reader (Thermo, United States) was used to measure the absorbance value at 450 nm.

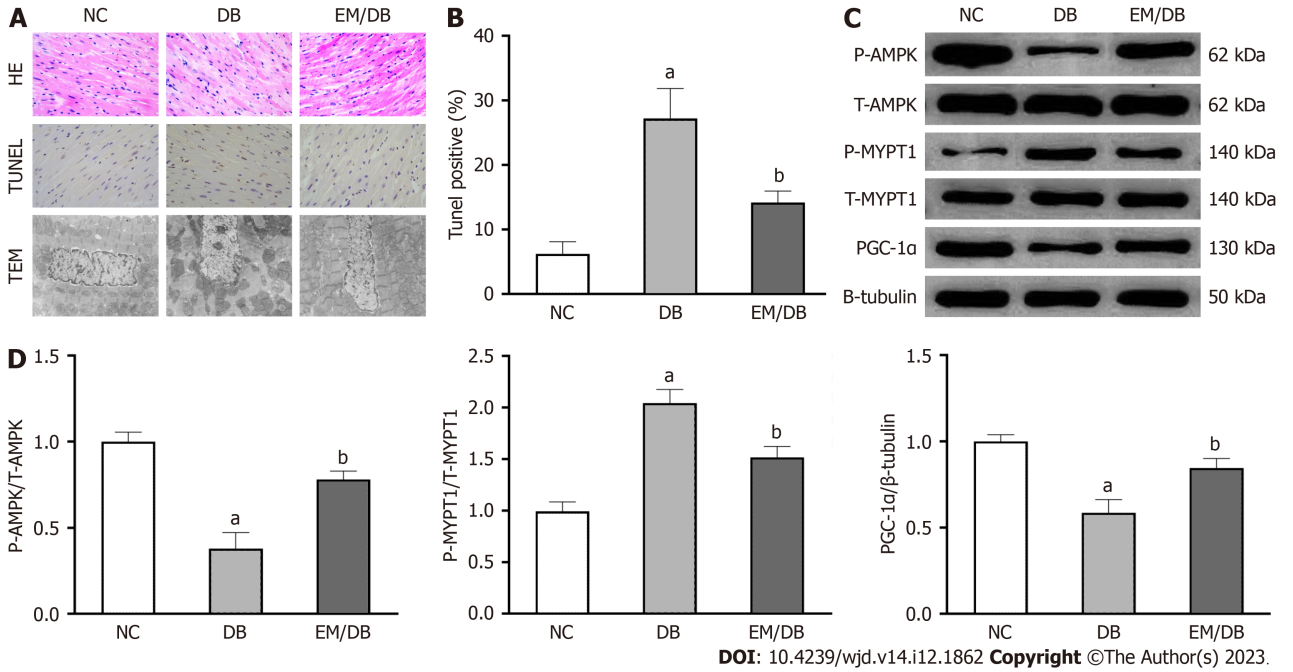

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Figure 2 Effects of empagliflozin on mitochondrial injury, apoptosis, and AMP-activated protein kinase, peroxisome proliferator-activated receptor-γ coactivator-1α, and the RhoA/ROCK pathway in db/db mice. A: Left ventricular sections stained with hematoxylin and eosin and by TdT-mediated dUTP-biotin nick end labeling to assess cardiomyocyte apoptosis: The nuclei of normal cardiomyocytes were blue while the nuclei of apoptosis-positive cardiomyocytes were brown. Transmission electron micrographs showed the effects of empagliflozin on the ultrastructure of the myocardium in db/db mice; B: Quantitation of apoptotic cells; C and D: The phosphorylation of AMP-activated protein kinase and myosin phosphatase target subunit 1, as well as the protein expression of peroxisome proliferator-activated receptor-γ coactivator-1α were measured by Western blot in three groups. Bars indicate the mean ± SD from three independent experiments (n = 3). β-tubulin was set as a control for normalization. NC: Normal control mice; DB: Db/db mice; EM/DB: Db/db mice treated with empagliflozin; HE: Hematoxylin and eosin; TEM: Transmission electron micrographs; p-AMPK: Phosphorylated AMP-activated protein kinase; p-MYPT1: Phosphorylated myosin phosphatase target subunit 1; TUNEL: TdT-mediated dUTP-biotin nick end labeling. $^{a}P < 0.05$ *vs* NC; $^{b}P < 0.05$ *vs* DB.

Flow cytometry

After digestion with 0.25% trypsin, the cardiomyocytes were collected and supplemented with Annexin V and propidium iodide (PI) binding buffer in the dark at 4 °C for 30 min. Annexin V and PI are used to distinguish between apoptotic and necrotic cells, and positive Annexin V can be labeled with fluorescent dye to identify early apoptosis. Flow cytometry (Beckman FC500, California, United States) was used to collect and analyze the cells. The early apoptotic cells were located in the upper right quadrant (Annexin V+/PI-).

TUNEL staining

A TUNEL assay kit (Beyotime, C1086-20T) was used to measure the apoptosis rates of cardiomyocytes in line with the manufacturer's instructions. The cell slides containing the cells were supplemented with 50 μl TUNEL reaction mixture for 2 h at 37 °C to identify apoptotic cells. The DAPI staining solution was then placed on slides in the dark at 37 °C for 20 min to mark the total cells. An inverted fluorescence microscope (Olympus, Japan) was used to observe the cell slides and take photos. The apoptotic index was calculated as the percentage of TUNEL-positive cells. Ten representative fields were evaluated for each group and the average value was calculated.

Intracellular ROS level determination

The fluorescent probe dichlorodihydrofluorescein (DCFH) diacetate (DCFH-DA, Beyotime, S0033) was used to detect the levels of ROS generation. Intracellular esterases convert DCFH-DA to DCFH, which is oxidized to fluorescent dichlorofluorescein (DCF) by an oxidant. The cells were cultured with 3 μM DCFH-DA in serum-free DMEM for 20 min at room temperature. A fluorescence microscope was used to observe cellular ROS and take photos. The changes in fluorescence were analyzed by the ImageJ program (Bio-Rad, California, United States).

Measurement of cellular superoxide dismutase activity

The activity of superoxide dismutase (SOD) in cardiomyocytes was determined with a kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China).

Mitochondrial membrane potential assay

JC-1, which produces red fluorescence in normal mitochondria, changes to green fluorescence with loss of mitochondrial membrane potential (MMP). The change in MMP was determined by the decrease in the red to green fluorescence ratio. The myocardial cells were cultured with JC-1 solution (Beyotime, C2006) at 25 °C for 20 min. The samples were detected

using a flow cytometer (Beckman FC500, California, United States) within 30 min.

Cellular ATP measurement

An ATP bioluminescence kit (Beyotime, S0026) was used to assay the ATP levels in isolated myocardial cells. Briefly, after drug intervention, the cells were collected, lysed, and centrifuged (14000 rpm for 8 min). Next, the firefly luciferase reagent, which emits light in the presence of ATP, was added to the supernatant. The bioluminescence signals were detected using a luminometer (Promega, United States). The concentration of ATP in the sample was calculated from the standard curve.

Real-time quantitative PCR analysis

The myocardial gene expression of *Bcl-2* and *Bax* was determined using real-time quantitative PCR. Trizol reagent (ThermoScientific, United States) was used to separate total RNA in cardiomyocytes. The experimental steps were carried out according to the manufacturer's procedure. The primers were provided by Sangon Biotechnology (Shanghai, China). Their sequences are as follows:

Bax: Forward, 5′-AGACACCTGAGCTGACCTTGGAG-3′ and reverse, 5′-TTCATCGCCAATTCGCCTGAGAC-3′; *Bcl-2*: Forward, 5′-TGGAGAGCGTCAACAGGGAGATG-3′ and reverse, 5′-GTGCAGATGCCGGTTCAGGTAC-3′; 18S rRNA: Forward, 5′-TGCGGAAGGATCATTAACGGA-3′ and reverse, 5′-AGTAGGAGAGGAGCGAGCGACC-3.

The Ct value of the target mRNA was calculated as follows: ΔCt = Ct purpose − Ct internal reference, and the relative expression levels of the target mRNA were decided by $2^{-\Delta\Delta Ct}$.

Western blot analysis

The total proteins of myocardial tissues and cardiomyocytes were lysed using RIPA lysate (Solarbio, R0010). The bicinchoninic acid protein assay (Solarbio, PC0020) was used to measure the concentration of proteins in the supernatant. The protein samples (50 μg, 15 μL) were then run on a 10% SDS-PAGE gel and subsequently blotted to a PVDF membrane (Millipore, Billerica, MA, United States, IPVH 0010). After being blocked with 5% nonfat dried milk, the membrane was incubated at 4 °C overnight with primary antibodies, followed by incubation with the goat anti-rabbit IgG as secondary antibody at 37 °C for 1.5 h. The antibodies used were as follows: Anti-phosphorylated myosin phosphatase target subunit 1 (p-MYPT1) and anti-total myosin phosphatase target subunit 1 (t-MYPT1), anti-cleaved caspase 3, anti-AMPK, anti-p-AMPK, anti-PGC-1α (these antibodies were all from Cell Signaling Technology, USA), anti-SGLT1 (Abcam, United States), anti-SGLT2 (Abcam), and anti-β-Tubulin (Abways). Finally, the membrane was detected using chemiluminescent reagents (Solarbio, SB-WB012S) and Image J (Bio-Rad).

Statistical analysis

Data are expressed as the mean ± SD. All data were verified to be normally distributed. One-way analysis of variance was used to detect the differences among multiple groups followed by the Tukey test if F was significant. The differences between the two groups (SGLT1 and SGLT2 protein) were determined using the Student's t-test. $P < 0.05$ was considered statistically significant. Data were analyzed using GraphPad Prism.9.0 software (GraphPad, CA, United States).

RESULTS

Effects of empagliflozin on biochemical and physiological parameters in DCM mice

Diabetic mice exhibited higher levels of FBG, HbA1c, FINS, HOMA-IR, and TC and the high ratio of heart weight to body weight (HW/BW) than control mice (Table 1) ($P < 0.05$). Among the three groups, no significant differences were observed in SABP. Empagliflozin treatment resulted in a significant reduction in FBG, HbA1C, FINS, and HOMA-IR levels in diabetic mice ($P < 0.05$). However, HW/BW and TC in diabetic mice were not altered by empagliflozin treatment.

Effects of empagliflozin on cardiac function in DCM mice

Compared with control mice, LVEF and LVFS were significantly decreased whereas E/A was enhanced in diabetic mice (Figure 1) ($P < 0.05$). Empagliflozin treatment substantially enhanced cardiac diastolic and systolic function by reducing E/A and increasing LVEF and LVFS in diabetic mice ($P < 0.05$).

Effects of empagliflozin on cardiac mitochondrial injury and apoptosis in DCM mice

HE histological staining revealed a well-organized arrangement of myocardial fibers in control mice (Figure 2A). In diabetic mice, the myocardial fibers were disorganized. Nevertheless, empagliflozin treatment improved myocardial tissue fiber arrangement in diabetic mice. Similarly, TEM examination of the myocardium ultrastructure indicated that mitochondria in control mice were longitudinally arranged between the muscle bundles, and their membrane structures remained intact. However, in diabetic mice, mitochondria exhibited partial disappearance, swelling, fragmentation, and pyknosis. The morphology of mitochondria in diabetic mice was partially restored by empagliflozin treatment. TUNEL staining revealed that cellular apoptosis in diabetic mice was increased compared with that in the NC group, which was reduced by empagliflozin treatment.

Table 1 Metabolic and physiological parameters in mice

	NC (*n* = 11)	DB (*n* = 7)	EM/DB (*n* = 7)
FBG (mmol/L)	4.98 ± 0.82	33.51 ± 2.96[1]	19.26 ± 3.12[2]
HbA1c (%)	4.47 ± 0.14	9.15 ± 1.16[1]	6.86 ± 0.81[2]
INS (mU/L)	82.88 ± 17.85	325.80 ± 47.30[1]	176.30 ± 30.89[2]
HOMO-IR	19.22 ± 5.76	472.60 ± 83.22[1]	190.50 ± 113.50[2]
TC (mmol/L)	2.57 ± 0.30	4.27 ± 0.18[1]	4.27 ± 0.27[1]
HW/BW (10^{-3})	5.21 ± 0.17	5.77 ± 0.12[1]	5.78 ± 0.09[1]
SABP (mmHg)	122.70 ± 2.37	122.90 ± 3.71	123.60 ± 5.03

[1]*P* 0.05 *vs* normal control mice.
[2]*P* 0.05 *vs* db/db mice.
Data are presented as the mean ± SD. NC: Normal control mice; DB: Db/db mice; EM: Empagliflozin; FBG: Fasting blood glucose; INS: Serum insulin; HW/BW: Ratio of heart weight to body weight; SABP: Systolic arterial blood pressure.

Effects of empagliflozin on AMPK, PGC-1α, and the RhoA/ROCK pathway in DCM mice

Phosphorylation of AMPK and MYPT1 represents the activation of AMPK and the RhoA/ROCK pathway, respectively. Herein, the protein levels of p-AMPK and PGC-1α in diabetic mice were significantly decreased compared with those in control mice ($P < 0.05$). The treatment of empagliflozin substantially increased the levels of p-AMPK and PGC-1α ($P < 0.05$). Meanwhile, the level of p-MYPT1 was significantly increased in diabetic mice compared with control mice ($P < 0.05$), which was notably inhibited by empagliflozin treatment ($P < 0.05$). These findings indicate that empagliflozin is associated with AMPK/PGC-1α and the RhoA/ROCK pathway in the myocardium of diabetic mice (Figure 2B and C).

Effects of empagliflozin concentrations on cardiomyocyte viability exposed to HG

HG significantly decreased cardiomyocyte viability (Figure 3A). Under HG conditions, empagliflozin concentrations ranging from 0.1 to 10 μM increased cell viability in a dose-dependent manner ($P < 0.05$), whereas 0.01 μM empagliflozin did not induce any changes in the viability of cells exposed to HG. The viability of cardiomyocytes treated with empagliflozin at concentrations between 10 and 20 μM did not show a significant difference. In addition, hyperosmosis did not affect the viability of these cells. Consequently, a 10 μM concentration of empagliflozin was chosen for subsequent experiments.

Effects of empagliflozin, FA, and PGC-1α on HG-induced cardiomyocyte apoptosis

Cell apoptosis was enhanced in HG conditions and was mitigated by empagliflozin, FA, and overexpression of PGC-1α protein ($P < 0.05$) (Figure 3B-D). Under the HG condition, the mRNA expression of the apoptosis-related gene *Bax* was upregulated ($P < 0.05$), whereas that of the anti-apoptosis indicator *Bcl-2* was downregulated ($P < 0.05$). Furthermore, the level of active caspase-3 protein was upregulated in cells exposed to HG ($P < 0.05$) (Figure 4). However, treatment with empagliflozin, FA, and the overexpression of PGC-1α protein reversed all these changes ($P < 0.05$). The addition of CC, an AMPK inhibitor, demonstrated the opposite effects of empagliflozin on cardiomyocytes.

Effects of empagliflozin, FA, and PGC-1α on HG-induced mitochondrial injury and oxidative stress in cardiomyocytes

Cellular ATP production and MMP reflect mitochondrial function. In this study, MMP and ATP levels in cardiomyocytes exposed to HG were substantially decreased. Furthermore, compared with the NG group, HG reduced the antioxidant enzyme SOD and enhanced cellular ROS levels ($P < 0.05$). Treatment with empagliflozin or FA or the overexpression of PGC-1α substantially increased MMP, ATP, and SOD levels, and reduced ROS production in cells exposed to HG (Figure 5). The addition of CC to the EM group weakened these effects ($P < 0.05$). These findings indicate that the activation of AMPK, upregulation of PGC-1α, or inhibition of the RhoA/ROCK pathway can enhance mitochondrial function and mitigate oxidative stress in HG-induced cardiomyocytes.

Relationship of empagliflozin with AMPK, PGC-1α, and the RhoA/ROCK pathway in cardiomyocytes in HG

As illustrated in Figure 6A-D, the protein levels of p-AMPK and PGC-1α were significantly reduced in cells exposed to HG compared with the NG group ($P < 0.05$), whereas the level of p-MYPT1 was increased ($P < 0.05$). Compared with the HG group, empagliflozin treatment upregulated the protein levels of p-AMPK and PGC-1α in cells exposed to HG ($P < 0.05$), accompanied by the downregulation of p-MYPT1 ($P < 0.05$), which was weakened by the addition of CC. In cells exposed to HG, the addition of FA and the overexpression of PGC-1α significantly upregulated PGC-1α protein level ($P < 0.05$). These findings indicate that empagliflozin can activate AMPK, inhibit the RhoA/ROCK pathway, and induce PGC-1α expression.

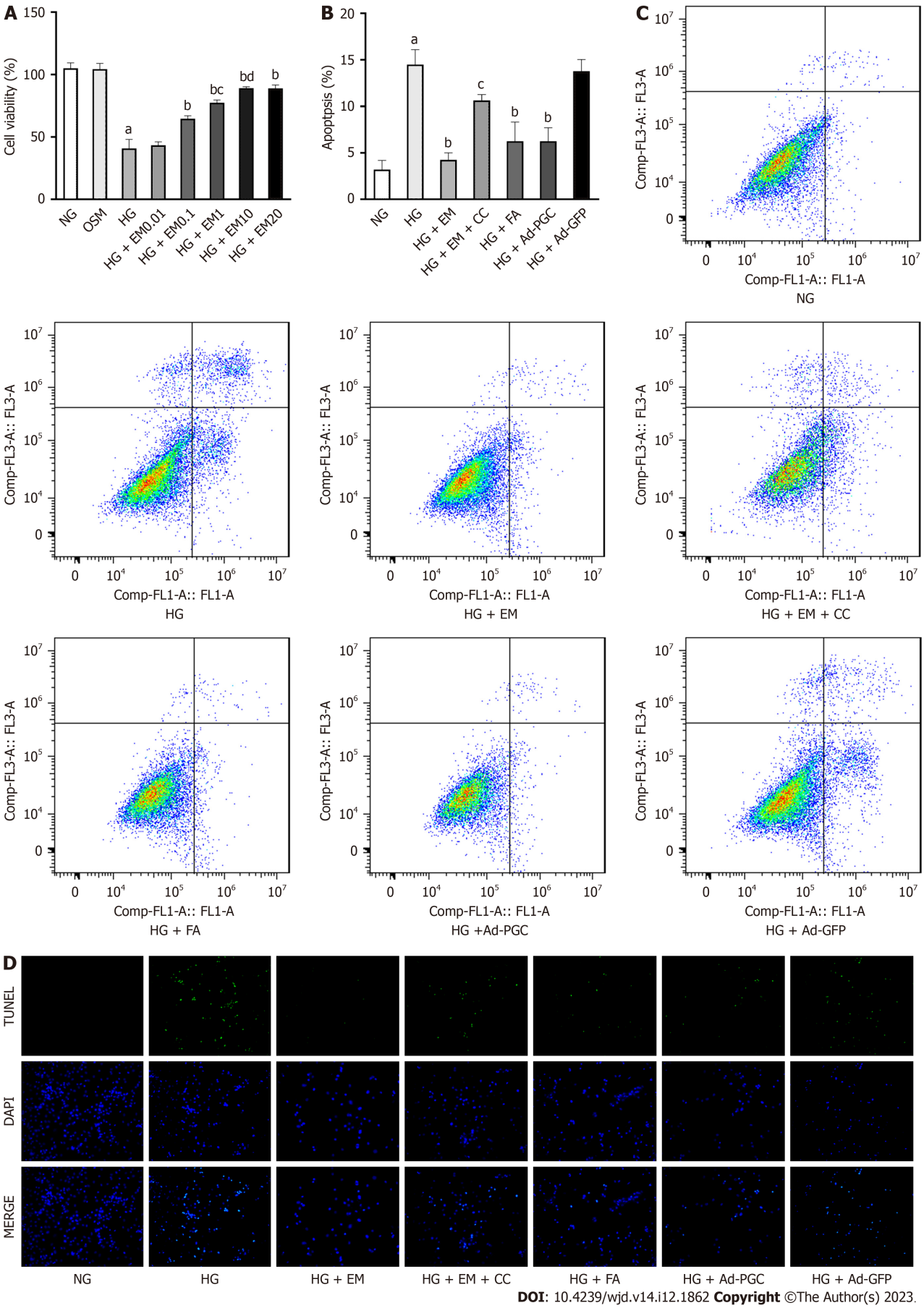

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Figure 3 Effects of empagliflozin, fasudil, and overexpression of peroxisome proliferator-activated receptor-γ coactivator-1α on high glucose-induced cardiomyocyte apoptosis *in vitro*. A: Effects of different concentrations of empagliflozin on viability of cardiomyocytes exposed to high

glucose (HG); B and C: Cardiomyocyte apoptosis measured by flow cytometry; D: TdT-mediated dUTP-biotin nick end labeling staining of cardiomyocytes in each group. Bars indicate the mean ± SD from three independent experiments (n = 3). NG: Normal glucose; HG: High glucose; EM: Empagliflozin; CC: Compound C; FA: Fasudil; HG + Ad–PGC: Cells were transfected with peroxisome proliferator-activated receptor-γ coactivator-1α-GFP-Ad; HG + Ad-GFP: Cells transfected with GFP-Ad. $^{a}P < 0.05$ *vs* NG; $^{b}P < 0.05$ *vs* HG; $^{c}P < 0.05$ *vs* HG + EM.

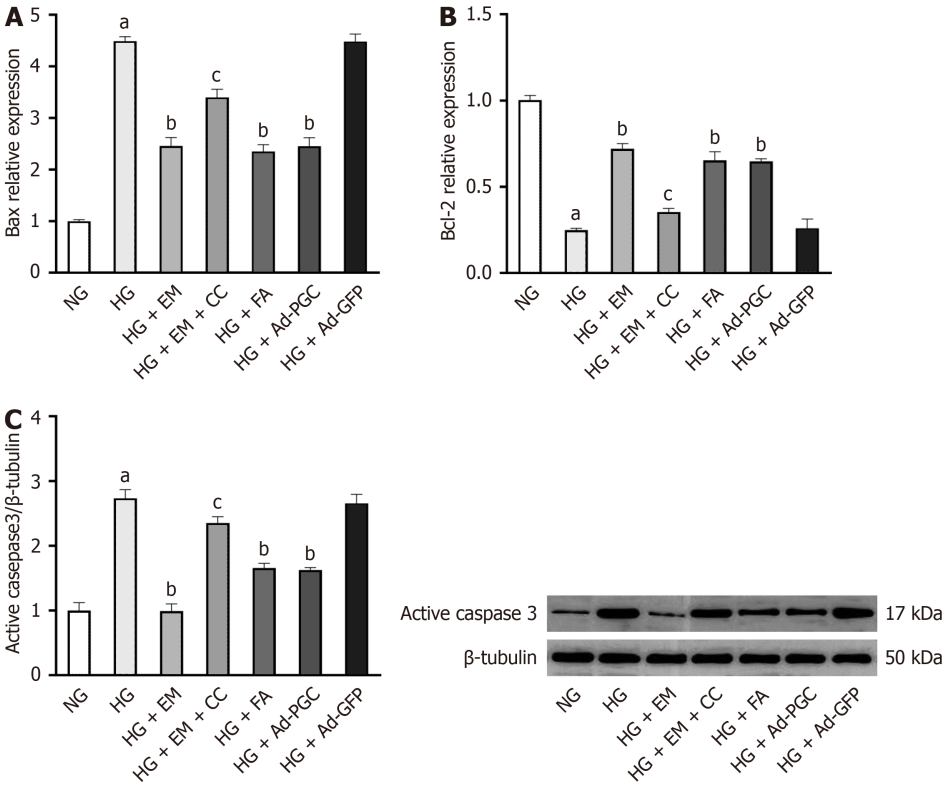

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Figure 4 Effects of empagliflozin, fasudil, and overexpression of peroxisome proliferator-activated receptor-γ coactivator-1α on apoptotic genes and related proteins *in vitro*. A and B: *Bax* and *Bcl-2* mRNA expression was quantified using real-time quantitative PCR in cardiomyocytes; C: Protein expression of active caspase-3 measured by Western blot. Bars indicate the mean ± SD from three independent experiments (n = 3). β-tubulin was set as a control for normalization. NG: Normal glucose; HG: High glucose; EM: Empagliflozin; CC: Compound C; FA: Fasudil; HG + Ad–PGC: Cells were transfected with peroxisome proliferator-activated receptor-γ coactivator-1α-GFP-Ad; HG + Ad-GFP: Cells transfected with GFP-Ad. $^{a}P < 0.05$ *vs* NG; $^{b}P < 0.05$ *vs* HG; $^{c}P < 0.05$ *vs* HG + EM.

SGLT1 and SGLT2 protein expression in cardiomyocytes

SGLT1 protein was expressed in cardiomyocytes. In cardiomyocytes under HG conditions, the protein expression of SGLT1 was significantly increased compared with the NG group. However, the SGLT2 protein was not expressed in cardiomyocytes under either the NG or HG conditions. (Figure 6E).

DISCUSSION

Db/db mice are typically employed as an animal model of type 2 DM (T2DM). In this study, db/db mice exhibited hyperglycemia, hyperlipidemia, and insulin resistance, which are consistent with the characteristics of T2DM. The effects of empagliflozin on cardiac function in db/db mice and HG-treated cardiomyocytes were evaluated and four main findings were demonstrated: (1) Db/db mice developed DCM, and hyperglycemia led to mitochondrial injury and increased cardiomyocyte apoptosis *in vivo* and *in vitro*; (2) Empagliflozin enhanced cardiac function in db/db mice and prevented mitochondrial injury, oxidative stress, and cardiomyocyte apoptosis *in vivo* and *in vitro*; (3) These protective effects of empagliflozin on myocardial cells were achieved through the activation of AMPK, inhibition of the RhoA/ROCK pathway, and upregulation of PGC-1α. AMPK could regulate the RhoA/ROCK pathway and PGC-1α expression, with PGC-1α being a downstream target of the RhoA/ROCK pathway; and (4) Cardiomyocytes expressed SGLT1 protein, but did not express SGLT2 protein in either NG or HG conditions.

DCM can occur in the early stage of DM, manifest as diastolic and systolic dysfunction, and eventually progress to decompensated HF, which results in increased mortality in patients with DM[2]. Our *in vivo* study demonstrated that at the age of 20 wk, db/db mice developed DCM, which was consistent with the findings of Lew *et al*[24]. Empagliflozin prevented DCM by enhancing impaired heart diastolic and systolic functions in db/db mice. Hammoudi *et al*[25] de-

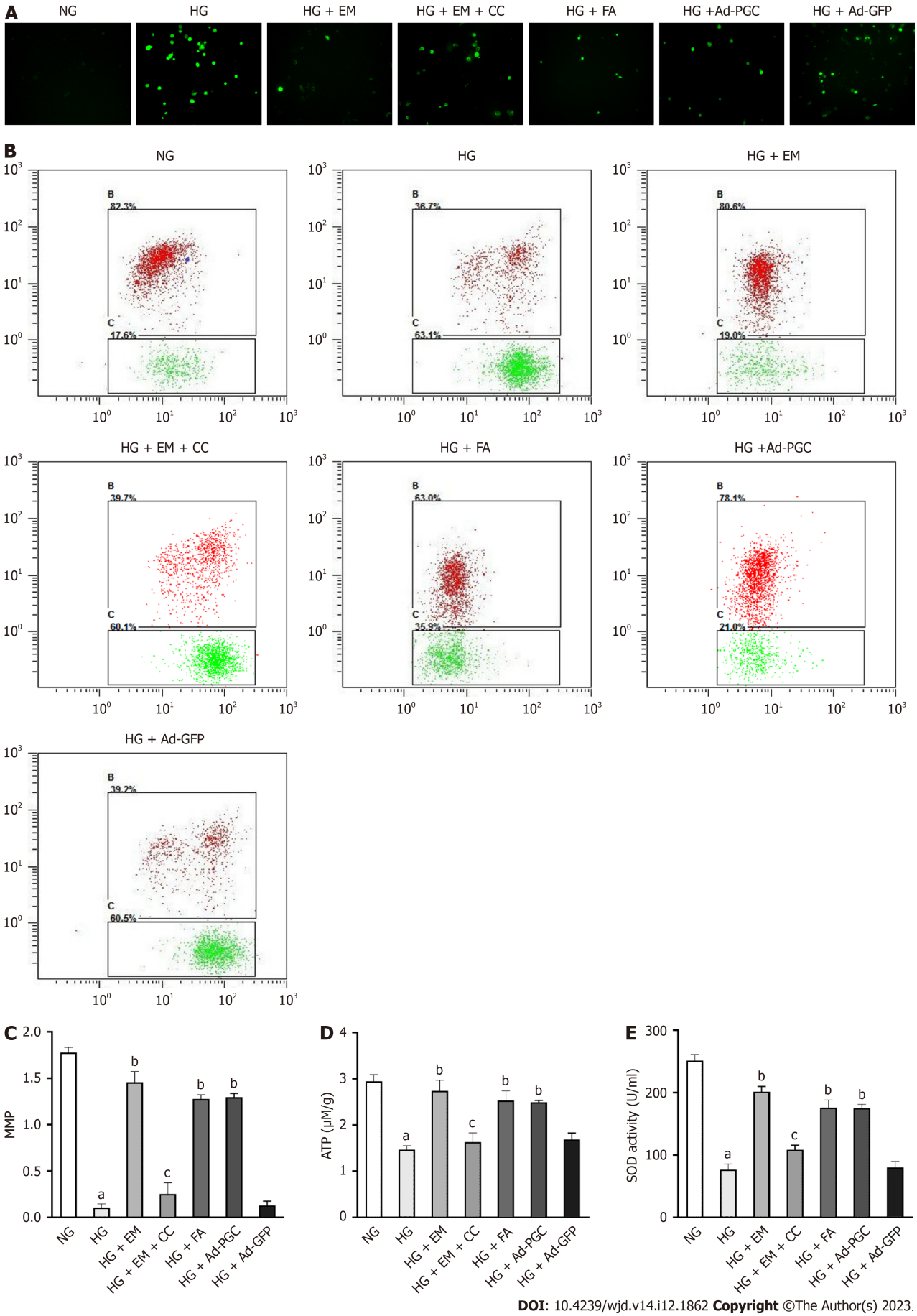

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Figure 5 Effects of empagliflozin, fasudil, and overexpression of peroxisome proliferator-activated receptor-γ coactivator-1α on high

glucose-induced mitochondrial injury and oxidative stress *in vitro*. A: Levels of intracellular high glucose; B and C: Changes in mitochondrial membrane potential; D: Cellular ATP production; E: Cellular superoxide dismutase activity. Bars indicate the mean ± SD from three independent experiments (n = 3). ROS: Reactive oxygen species; MMP: Mitochondrial membrane potential; SOD: Superoxide dismutase; NG: Normal glucose; HG: High glucose; EM: Empagliflozin; CC: Compound C; FA: Fasudil; HG + Ad–PGC: Cells were transfected with peroxisome proliferator-activated receptor-γ coactivator-1α-GFP-Ad; HG + Ad-GFP: Cells transfected with GFP-Ad. [a]P < 0.05 *vs* NG; [b]P < 0.05 *vs* HG; [c]P < 0.05 *vs* HG + EM.

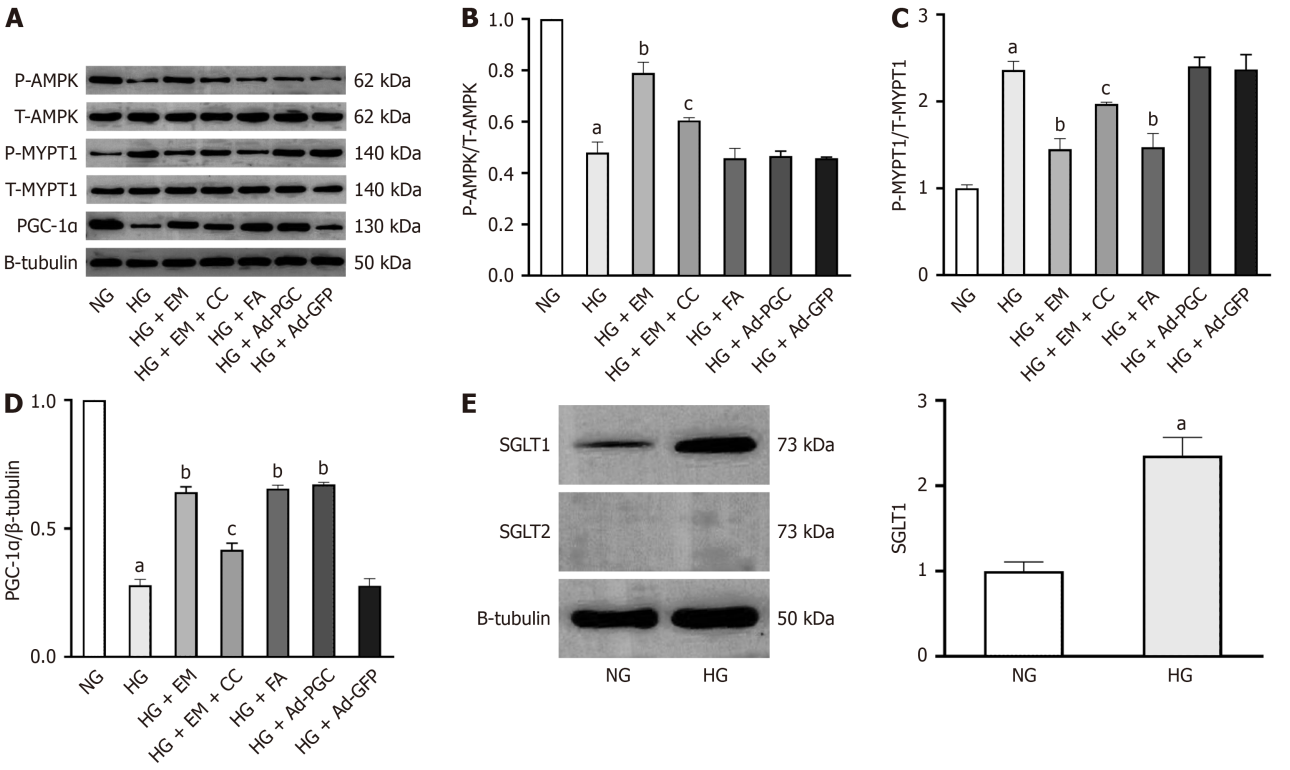

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Figure 6 Relationship of empagliflozin with AMP-activated protein kinase, peroxisome proliferator-activated receptor-γ coactivator-1α, and the RhoA/ROCK pathway in cardiomyocytes in HG conditions *in vitro*. A-D: The phosphorylation of AMP-activated protein kinase and myosin phosphatase target subunit 1, as well as the protein expression of peroxisome proliferator-activated receptor-γ coactivator-1α were measured by Western blot in three groups; E: Sodium-glucose cotransporter (SGLT)1 and SGLT2 protein expression in cardiomyocytes. Bars indicate the mean ± SD from three independent experiments (n = 3). β-tubulin was set as a control for normalization. NG: Normal glucose; HG: High glucose; EM: Empagliflozin; CC: Compound C; FA: Fasudil; HG + Ad–PGC: Cells were transfected with PGC-1α-GFP-Ad. [a]P < 0.05 *vs* NG; [b]P < 0.05 *vs* HG; [c]P < 0.05 *vs* HG + EM.

monstrated that treatment with empagliflozin at a dose of 10 mg/kg/d for 6 wk enhanced diastolic function and contractile reserve instead of systolic function in ob/ob mice. The varied effects of empagliflozin on cardiac systolic function may be attributed to the differences in the animal models used. Furthermore, empagliflozin reduced hyperglycemia and insulin resistance in db/db mice, but did not affect serum lipid, BW, or SABP levels, which was consistent with the findings of a previous study[26]. However, these mild metabolic enhancements cannot simply explain the substantial benefits of SGLT2 inhibitors in DCM.

Packer concluded that the cardiovascular advantages of SGLT2 inhibitors could not be attributed to the control of hyperglycemia, ketogenesis, and natriuretic action, but might be associated with the stimulation of autophagy and reduction of intracellular sodium in the myocardium[27]. Remarkably, several studies have demonstrated that SGLT2 inhibitors have numerous mechanisms of action on DCM, including the regulation of cardiac iron homeostasis, anti-inflammation, anti-fibrosis, and anti-oxidative stress[28]. Cardiomyocytes were cultured *in vitro* to clarify the direct effects of empagliflozin on the myocardium, independent of metabolic improvements. First, the protein expression of SGLT1 and SGLT2 in isolated myocardial cells was investigated, indicating that SGLT2 protein was not expressed in primary cardiomyocytes under either NG or HG conditions. This finding is consistent with the results of Di Franco *et al* [29], who reported that SGLT2 was not expressed in the human myocardium under normal or pathological conditions. Similarly, Mustroph *et al*[30] reported that SGLT2 expression was not identified in healthy or failing myocardium in humans and mice. Therefore, there may be a direct effect that elucidates the protection offered by empagliflozin to the myocardium independent of the SGLT-2 protein.

Accumulating evidence demonstrated that hyperglycemia and increased cardiac lipid deposition increase cell ROS generation[31]. This increased ROS generation can trigger oxidative stress, disrupt mitochondrial structure, and result in mitochondrial dysfunction in the heart of diabetic patients, which contributes to myocardial cell apoptosis and necrosis

[32]. In this study, alterations in mitochondrial morphology were observed in db/db mice, which were mitigated by empagliflozin treatment. HG-induced injury to mitochondria results in the generation of less ATP, decreased MMP, excess ROS, and decreased SOD. In other words, HG triggers oxidative stress, where empagliflozin treatment prevented mitochondrial injury and oxidative stress in myocardial cells. The role of mitochondrial function in maintaining cardiac function is crucial. Empagliflozin was linked to the enhancement of mitochondrial biogenesis and energetics, ultimately resulting in the suppression of cardiac remodeling and dysfunction[33-35]. DCM mice exhibited enhanced myocardial apoptosis, which was closely associated with mitochondrial injury and oxidative stress[36]. This study also demonstrated obvious apoptotic cells in the myocardium exposed to HG *in vivo* and *in vitro*, and empagliflozin mitigated HG-induced cardiac cell apoptosis by regulating mitochondria-dependent apoptosis pathways, which was consistent with the findings of a previous study[37].

Some related molecular pathways have been discussed to elaborate the protection mechanism of empagliflozin against cardiomyocytes. AMPK plays a crucial role in cardioprotection[38]. HG suppressed AMPK activity and increased mitochondrial dysfunction and apoptosis of cardiomyocytes, and AMPK activation ablated hyperglycemia-induced cardiac oxidative stress, mitochondrial injury, and myocardial apoptosis, ultimately resulting in enhanced cardiac function in DCM mice[39-41]. Consistent with these findings, our study indicated that empagliflozin increased AMPK activity, and the activation of AMPK resulted in anti-oxidative stress and anti-apoptosis effects on cardiomyocytes. Furthermore, our findings indicated that AMPK could regulate the RhoA/ROCK pathway and PGC-1α expression. It is well-established that AMPK activation results in the suppression of the RhoA/ROCK pathway in AngII-induced human vascular smooth muscle cells[42]. The RhoA/ROCK pathway plays a crucial role in the development of diabetic complications[43]. The inhibition of ROCK reduced HG/lipopolysaccharide-induced myocardial apoptosis and mitochondrial damage through the activation of autophagy[44,45]. The findings of this study demonstrated that inhibiting the RhoA/ROCK pathway prevented oxidative stress and apoptosis of cardiomyocytes under HG conditions, and enhanced mitochondrial dysfunction, which was consistent with the findings of our previous study[21]. In addition, the activation of AMPK could directly phosphorylate PGC-1α in skeletal muscle, or indirectly regulated PGC-1α expression through SIRT1 activation[46,47]. It has been confirmed that ROCK suppression can induce PGC-1α expression in rat striatal neurodegeneration[48]. In a rat model of T2DM, PGC-1α is closely associated with mitochondrial biogenesis and atrial remodeling, and empagliflozin can enhance impaired mitochondrial biogenesis and mitochondrial dysfunction, while also improving atrial remodeling through upregulation of PGC-1α[49]. Similarly, in DCM rats, the restoration of PGC-1α can enhance mitochondrial damage and cardiac dysfunction[50]. Our study demonstrated that empagliflozin upregulated PGC-1α expression under HG conditions, and inhibited cardiomyocyte oxidative stress, mitochondrial injury, and apoptosis. PGC-1α expression was modulated in part by AMPK and the RhoA/ROCK pathway.

To date, there has been few studies on the effects of empagliflozin on HG-induced cardiomyocyte apoptosis and the underlying mechanism. Cardiomyocyte apoptosis is believed to be the initial factor contributing to HF in DCM[6]. This study offers a novel molecular foundation for the anti-HF effects of empagliflozin. However, this study has some limitations: (1) Db/db mice exhibited hyperglycemia with hyperlipidemia, but the *in vitro* experiments only employed HG without the addition of palmitate. Consequently, the cellular models did not precisely match the animal models; and (2) No positive drug control was established in *in vivo* experiments. Further appropriate study should be conducted to solve these limitations.

CONCLUSION

In conclusion, this study demonstrated that empagliflozin not only controlled glycemic changes but also improved mitochondrial injury and cardiac dysfunction in db/db mice. HG-induced oxidative stress and cardiomyocyte apoptosis were mitigated by empagliflozin through the activation of AMPK/PGC-1α and inhibition of the RhoA/ROCK pathway. These findings demonstrated that empagliflozin exerted direct beneficial effects on cardiomyocytes independent of SGLT2 inhibition.

ARTICLE HIGHLIGHTS

Research background

Diabetic cardiomyopathy (DCM) increases the risk of hospitalization for heart failure in diabetic patients. However, there is no specific therapy to delay the progression of DCM. Empagliflozin has been confirmed to reduce the risk of hospitalization for heart failure in diabetic patients. However, the molecular mechanisms by which these agents exert cardioprotection remain unclear.

Research motivation

To explore the effects of empagliflozin on the development of DCM.

Research objectives

To investigate whether empagliflozin can improve mitochondrial injury and cardiac dysfunction, and prevent high glucose (HG)-induced oxidative stress and cardiomyocyte apoptosis, along with the underlying molecular mechanism.

Research methods

We used db/db mice and primary cardiomyocytes from neonatal rats stimulated with HG (30 mmol/L) separately as *in vivo* and *in vitro* models. Cardiac function was evaluated by echocardiography. We used transmission electron microscopy to observe mitochondrial injury. RT-qPCR, Western blot, flow cytometry, TdT-mediated dUTP-biotin nick end labeling staining, and immunofluorescence were used to investigate the effects of empagliflozin treatment on cellular processes in cardiomyocytes of neonatal rats stimulated with HG.

Research results

Empagliflozin significantly improved cardiac dysfunction and dramatically reduced myocardial apoptosis, accompanied by upregulation of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-γ coactivator-1 α (PGC-1α), as well as downregulation of myosin phosphatase target subunit 1 (MYPT1) in the heart of mice. At the cellular level, treatment of cardiomyocytes with empagliflozin or FA (a ROCK inhibitor) or overexpression of PGC-1α all markedly attenuated HG-induced mitochondrial injury, oxidative stress, and cardiomyocyte apoptosis. However, AMPK inhibitor reversed the above effects in part. Furthermore, no sodium-glucose cotransporter (SGLT)2 protein expression was detected in cardiomyocytes.

Research conclusions

Empagliflozin improves mitochondrial injury and cardiac dysfunction in db/db mice, and prevents HG-induced oxidative stress and cardiomyocyte apoptosis *in vitro* at least partially by activating AMPK/PGC-1α and inhibiting the RhoA/ROCK pathway independent of SGLT2.

Research perspectives

Next step, we will establish a positive drug control *in vivo* and further clarify the effects of empagliflozin on DCM, with an objective of providing a new strategy for the prevention and treatment of DCM.

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FOOTNOTES

Author contributions: Li N, Zhu QX, Li GZ, Wang T, and Zhou H designed and coordinated the study; Li N, Zhu QX, Li GZ, and Zhou H performed the experiments, and acquired and analyzed the data; Li N interpreted the data; Li N and Zhou H wrote the manuscript; all authors approved the final version of the article.

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CASE REPORT

Maturity-onset diabetes of the young type 10 caused by an Ala2Thr mutation of *INS*: A case report

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Abstract

BACKGROUND

Maturity-onset diabetes of the young 10 caused by the c.4G>A (p.Ala2Thr) mutation is extremely rare, with only two reported studies to date. Herein, we report another case that differs from previous cases in phenotype.

CASE SUMMARY

The proband developed diabetes at the age of 27 years, despite having a normal body mass index (BMI). She exhibited partial impairment of islet function, tested positive for islet antibodies, and required high doses of insulin. Her sister also carried the c.4G>A (p.Ala2Thr) mutation, and their mother was strongly suspected to carry the mutated gene. Her sister developed diabetes around 40 years of age and required high doses of insulin, while the mother was diagnosed in her 20s and was managed with oral hypoglycemic agents; neither of them were obese.

CONCLUSION

p.Ala2Thr mutation carriers often experience relatively later onset and normal BMI. Treatment regimens vary between individuals.

Key Words: Maturity-onset diabetes of the young 10; Insulin gene; Ala2Thr mutation; Case report

Core Tip: Maturity-onset diabetes of the young (MODY) 10 is uncommon, especially when caused by the c.4G>A (p.Ala2Thr) mutation, and thus, our knowledge of this disease is limited. Herein, we present an atypical MODY10 case resulting from the p.Ala2Thr mutation, which differs from previous reports and deviates from the prevalent phenotype of MODY. This patient exhibited insulin resistance and positive islet autoantibodies, as well as demonstrated significant familial inheritance and hearing impairment, which increased the potential for misdiagnosis.

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INTRODUCTION

Maturity-onset diabetes of the young (MODY) is an autosomal dominant monogenic diabetes, characterized by islet cell dysfunction or impaired insulin synthesis and secretion[1]. Most individuals have early age onset diabetes and usually do not require insulin during the early stages of the disease. MODY accounts for approximately 1%-5% of diabetes, but is often misdiagnosed as type 1 diabetes mellitus (T1DM) or type 2 diabetes mellitus for various reasons[2].

At least 14 subtypes of MODY have been identified[3]. MODY10 is relatively rare, and is caused by a mutation of the 11p15.5 site on chromosome 11 encoding insulin[4]. Preproinsulin is synthesized by the transcription and translation of *INS*, and subsequently cleaved to secrete insulin[5]. Therefore, *INS* mutations are strongly associated with abnormal insulin generation and glucose metabolism. Two studies have reported the c.4G>A (p.Ala2Thr) mutation in MODY10 patients and confirmed that this mutation was closely related to preproinsulin cleavage and insulin synthesis[6,7]. Here, we report another clinical case of MODY10 caused by the c.4G>A (p.Ala2Thr) mutation in a Chinese pedigree, and review the literature to summarize the clinical characteristics of MODY10 resulting from *INS* c.4G>A (p.Ala2Thr).

CASE PRESENTATION

Chief complaints

This case report describes a 53-years-old woman who had suffered from polyphagia, polydipsia, polyuria, and weight loss for 26 years, as well as repeated dizziness, cold sweats, and palpitations for one week.

History of present illness

Symptoms including recurrent dizziness, cold sweats, and palpitations started one week before the patient presented to the hospital. Blood glucose levels were often < 3.9 mmol/L during these episodes.

History of past illness

The individual presented with typical hyperglycemic symptoms and was diagnosed with T1DM in 1996 when she was 27 years old. Both fasting C-peptide (FCP) and postprandial C-peptide (PCP) levels were low, although details on the specific data were unavailable. Islet-related antibodies and hemoglobin A1c (HbA1c) levels could not be recalled. Due to an early age onset, as well as being non-obese and exhibiting pancreatic insufficiency, the patient was diagnosed with T1DM. Insulin therapy was initiated (12 U, 8 U, 8 U Novolin-R before three meals, 0.56 U/kg/d). Treatment regimens were subsequently adjusted according to the patient's blood glucose levels. After three years, the regimen was modified to Novolin-R 50/50 (18 U before breakfast and 12 U before dinner, 0.625 U/kg/d). However, since her blood glucose levels remained high, doses were gradually increased to 20 U and 18 U. Five years later, the proband's HbA1c levels were 6.8%, fasting blood glucose (FBG) levels were 5.32 mmol/L, and FCP levels were 430 pmol/L. The proband was positive for both glutamic acid decarboxylase antibody (GADA) and islet cell antibody. Despite the absence of foamy urine, the urine albumin-creatinine ratio was 240.90 mg/g and 214.19 mg/g, and the estimated glomerular rate (eGFR) was 71.59 mL/min/(1.73 m²). She was diagnosed with T1DM with diabetic kidney disease (DKD) (G2A2 stage). The patient exhibited higher blood glucose levels (10-12 mmol/L) after lunch and dinner, but fasting glucose (around 7 mmol/L) and post-breakfast glucose (around 8 mmol/L) levels were normal. The patient's treatment regimen was switched to Novolin 70/30, and gradually increased to 30 U before breakfast and 18 U before dinner (1 U/kg/d). Following this treatment, her FBG levels were 4.5-5 mmol/L, and 2 h postprandial blood glucose (PBG) levels were 6.7-7.8 mmol/L.

Twelve years after disease onset, the patient complained of numbness in her toes without pain and abnormal sweating. Electromyography revealed a decreased amplitude in her left superficial peroneal nerve. DKD progressed to G2A3 stage. Islet function appeared to be stable with FCP levels of 317 pmo1/L and PCP levels of 619 pmo1/L. Because of the high insulin dosage requirements and the absence of progressive pancreatic function decline, MODY was considered, and the patient began combined oral hypoglycemic therapy. Thus, the treatment regimen was switched to metformin [0.5 g ter in die (TID)], acarbose (50 mg TID) and insulin aspart 30 (20 U before breakfast and 10 U before dinner, 0.64 U/kg/d). Under this treatment regimen, the proband's HbA1c levels fluctuated between 6.8% and 8%.

In 2020, 24 years after disease onset, ultrasound doppler showed intima-media thickening in the carotid arteries and atherosclerotic plaques in multiple arteries. The patient suffered from fluctuating blood glucose levels and was frequently hypoglycemic. At this time, the hypoglycemic regimen was changed to metformin (0.5 g bis in die) combined with four daily insulin injections (4 U, 6 U, 5 U insulin aspart before three meals and 9 U insulin degludec before bedtime, 0.5 U/kg/d). Although the patient's HbA1c levels fluctuated between 7% and 9%, she often experienced hypoglycemia one hour after meals.

Personal and family history

The proband had a history of hypertension, dyslipidemia, Hashimoto's thyroiditis, bilateral sensorineural deafness (average hearing 50 dB), pre-excitation syndrome, and purpura nephritis (cured).

The proband's daughter was healthy. Her father was diagnosed with diabetes mellitus at 60-years-old. Her mother was thin and suffered from chronic kidney disease (diagnosed in her 20s), diabetes (diagnosed in her 30s), hearing loss (details unknown), and died of kidney failure at the age of 42 years. Her mother was insulin-independent. Details regarding the mother's diabetic complications are unclear, but it is known she never complained of numbness or pain, blurred vision, and abnormal sweating. The proband has two siblings: Her sister who was normal in size was diagnosed with diabetes around 40 years old, while her half-sister was healthy. The diabetic sibling suffered hearing loss and hypertension, but no diabetic complications. Her auto-antibodies and islet function were unknown and she was treated with insulin aspart 30 (a total dose of 27 U, 0.54 U/kg/d). The child of the diabetic sibling was healthy.

Physical examination

Physical examination revealed that her body mass index (BMI) was 20.24 kg/m^2, waist circumference was 75 cm, and waist-hip ratio was 0.91. No abnormal signs were found during cardiopulmonary and abdominal examinations, except for a surgical scar on her abdomen. Diabetic peripheral neuropathy (DPN) screening and dorsalis pedis pulsations on both sides were normal.

Laboratory examinations

The proband's HbA1c levels were 9.1%, FBG levels were 6.8 mmol/L, PBG levels were 21.8 mmol/L, FCP levels were 135.4 pmol/L, PCP levels were 600.1 pmol/L, Scr levels were 79 umol/L, eGFR levels were 70.18 mL/min/1.73 m^2, 24 h urine protein was 0.531 g, and lactic acid levels were 0.6mmol/L.

The proband and her sister have a heterozygous mutation (c.4G>A) in exon 2 of *INS* on chromosome 11, leading to the amino acid replacement p.Ala2Thr (A2T). Her father did not carry the mutation (Figure 1), and neither did her daughter. Due to early death, the mother did not undergo genetic testing (Figure 2). The proband's human leukocyte antigen (HLA) genotype was also evaluated, and no HLA gene variations linked to T1DM were found (Table 1). The proband and her family members did not grant consent for genetic testing of mitochondrial gene mutations.

Imaging examinations

The findings of the fundus examination were normal.

FINAL DIAGNOSIS

Combined with the genetic sequencing results, the proband was eventually diagnosed as MODY10, with the presence of DKD (G2A3 stage), DPN, and diabetic macroangiopathy.

TREATMENT

Subsequently, she was prescribed metformin (0.5 g before dinner) and four daily insulin injections (4 U, 4 U, 3 U insulin aspart before three meals and 14 U insulin degludec before bedtime, 0.52 U/kg/d).

OUTCOME AND FOLLOW-UP

The patient's blood glucose levels were tracked using a continuous glucose monitoring system. During the 9-d review period, she spent 42% of her time within 3.9-10 mmol/L, 50% of her time between 10.1-13.9 mmol/L, and 8% of her time within 3.1-3.8 mmol/L.

DISCUSSION

MODY is a type of diabetes that is caused by a single gene mutation and inherited in an autosomal dominant manner[1]. To date, at least 14 types of MODY have been identified (Table 2). The clinical features and treatment regimens of MODY patients vary not only by subtypes, but also within the same subtype[8,9]. Due to a limited number of reports on

Table 1 Human leukocyte antigen genotype of the proband

Gene	Allele1	Allele2
HLA-DRB1	DRB1 09:01	DRB1 09:01
HLA-DQA1	DQA1 03:03	DQA1 03:03
HLA-DQB1	DQB1 03:02	DQB1 03:03
HLA-A	A 02:01	A 24:02
HLA-B	B 51:01	B 51:01
HLA-C	C 01:02	C 01:02

HLA: Human leukocyte antigen.

Table 2 The clinical features of maturity-onset diabetes of the young patients

Subtype	Gene mutation	Prevalence	Clinical feature	Treatment
MODY1	HNF4A	Common	One-half of patients are neonatal macrosomia; blood sugar control deteriorates gradually as the disease advances; low levels of apolipoproteins and triglycerides; without insulin resistance or β cell autoimmunity	Medication-free in the early stage; sensitive to sulfonylureas
MODY2	GCK	Common	Slight elevation in fasting blood glucose and glycated hemoglobin levels; usually asymptomatic	Typically does not require medication
MODY3	HNF1A	Common	Renal glucose threshold is decreased; low levels of hs□CRP; without insulin resistance or β cell autoimmunity; similar to MODY1	Sensitive to sulfonylureas
MODY4	PDX1/IPF1	Rare	Overweight/obesity in some patients; commonly occurs post-puberty; postprandial blood sugar usually rises significantly	Mostly treated with insulin
MODY5	HNF1B	Uncommon	Often combined with genitourinary malformations, hepatic dysfunction, renal dysfunction, renal cysts, hyperuricemia, exocrine pancreas insufficiency; onset occurs typically during adolescence or early adulthood.	Early insulin therapy may be required
MODY6	NEUROD1	Rare	Phenotype is different. Overweight/obesity, intellectual disabilities and brain abnormalities occur in some patients	Significant variations in treatment regimens
MODY7	KLF11	Extremely rare	Mild hyperglycemia, hyperlipidemia	Insulin
MODY8	CEL	Extremely rare	Impaired endocrine and exocrine pancreatic function	Insulin
MODY9	PAX4	Extremely rare	Progressive hyperglycemia; ketoacidosis may occur	Mostly treated with insulin
MODY10	INS	Rare	Earlier onset of diabetes, an increased risk of diabetic microvascular complication; degree of islet dysfunction varies	Significant variations in treatment regimens
MODY11	BLK	Extremely rare	Overweight/obesity in some patients	Most patients require insulin, but some may be treated with diet or oral hypoglycemic agents
MODY12	ABCC8	Rare	Common in neonatal diabetes, symptoms are similar to MODY1 and 3	Sensitive to sulfonylureas
MODY13	KCNJ11	Extremely rare	Common in neonatal diabetes, some patients develop diabetes from the second decade of life onwards	Sensitive to sulfonylureas
MODY14	APPL1	Extremely rare	Overweight/obesity in some patients	Significant variations in treatment regimens

MODY: Maturity-onset diabetes of the young; CRP: High-sensitivity C-reactive protein.

MODY10, less is known about this subtype.

Genetic testing of the proband and her sister revealed an A2T mutation in INS, indicating that MODY10 should be considered. However, the patient tested positive for islet antibodies, necessitating differentiation from T1DM. Subsequent HLA gene testing conclusively excluded this possibility. Indeed, islet-related antibody positivity is not exclusive to T1DM. In a study by Urbanová *et al*[10] consisting of 28 MODY patients from the Czech Republic, seven individuals were found to be positive for GADA or islet antigen 2 antibody. Although it was not clear why these patients were positive, the existence of islet autoantibodies seems to be correlated with later onset and worsening glycemic control[10]. Despite this,

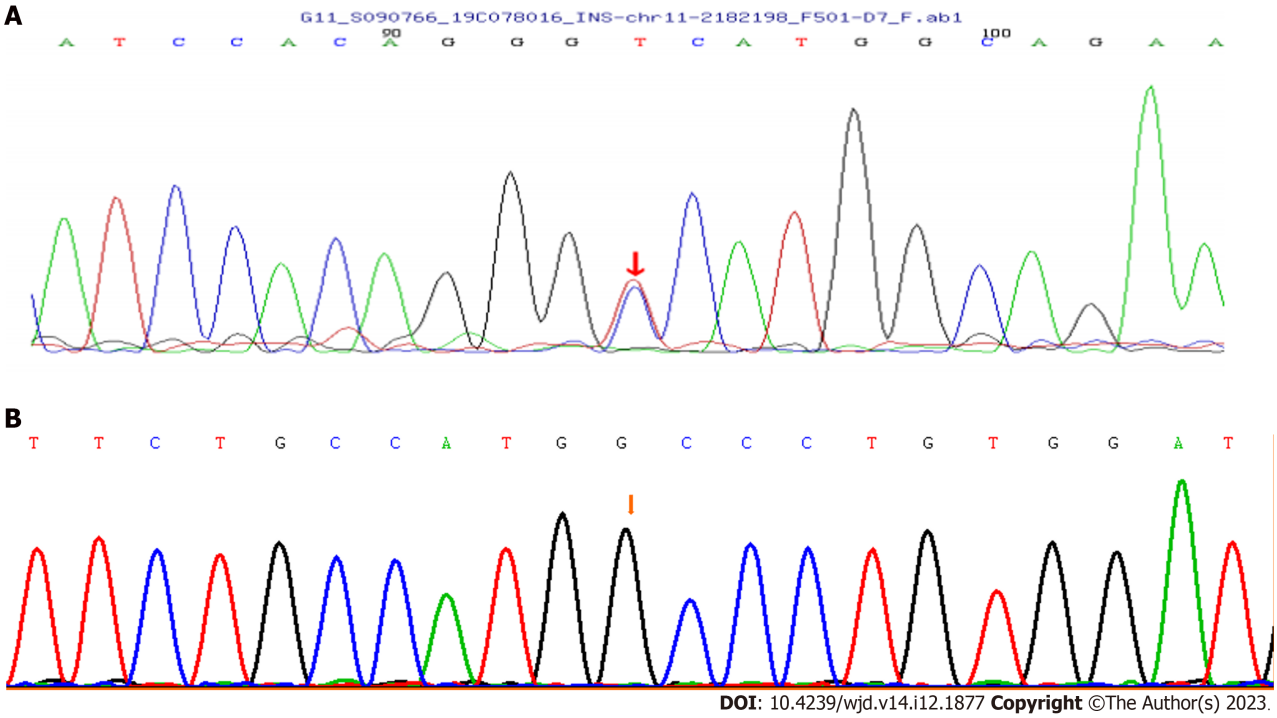

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Figure 1 *INS* gene sequence map of the proband and her father. A: Genetic testing data of the proband. The c.4G>A (p.Ala2Thr) mutation is shown by the red arrow; B: The genotype of this locus was normal in the father of the proband, which is shown by the black arrow.

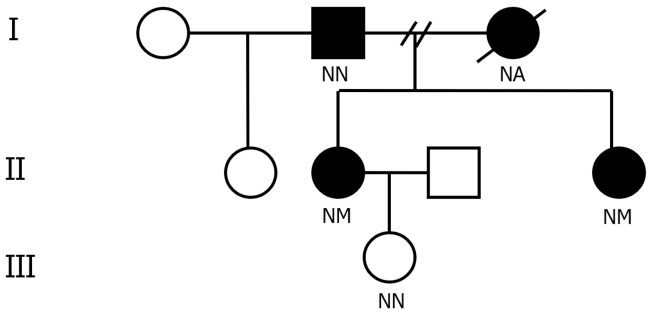

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Figure 2 The family pedigree. Participants with diabetes are shown in black. Women are represented by circles and men by squares. NA: Not tested; NM: Heterozygote; NN: Wild type.

the proband, her mother, and her diabetic sibling all suffered from diabetes and hearing impairment, prompting consideration of mitochondrial diabetes. However, the patient's lactate levels were normal, and a progressive decline in islet function was not observed. In addition, clinical features of mitochondrial diabetes, such as stroke, skeletal muscle impairment, or retinopathy, were not observed[11]. Furthermore, the offspring of the proband and her sister remained healthy. Considering these factors, the likelihood of mitochondrial diabetes was low. Multiple studies have reported that hearing impairment occurs in many non-mitochondrial diabetic patients, as well as within the MODY patient[12]. Hyperglycemia, microvascular complications, and mitochondrial damage are probably the main reasons for hearing loss in individuals with diabetes[12].

Based on the available literature, individuals with MODY10 tended to have an earlier onset of diabetes, with an average age of onset at 13.7 years, and were non-obese[13]. They were usually negative for islet antibodies and exhibited an increased risk of diabetic microvascular complications[5,8]. Due to differences in mutation sites, individuals with MODY10 exhibited varying degrees of islet dysfunction and required individualized treatment regimens[4,5,14,15]. Treatment options included diet and exercise, oral hypoglycemic agents, and insulin, with the highest insulin usage rate among them. Although patients can be treated with diet or oral hypoglycemic agents at diagnosis, they become insulin-independent as the condition progresses. In some cases, high doses of insulin supplementation might be necessary[5,8].

In our study, the proband and her sister were diagnosed with MODY10 and their mother was strongly suspected of having the disease. The clinical features of these three persons were consistent with some previous studies, but not all. Specifically, all three persons were non-obese and received different treatment regimens. Diabetic microangiopathy appeared to be more common than macroangiopathy. However, there were also some differences. Firstly, all the individuals in our study had a later age of onset, at least later than the common age of onset of MODY10[13]. Secondly,

Table 3 Clinical and biochemical parameters of all Ala2Thr mutation carriers

	Our study		Zhang *et al*[7]				Yan *et al*[6]					
No.	**1**	**2**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**5**	**6**
Age (yr)	53	48	25	46	42	69	/	47	66	58	34	62
Sex	Female	Female	Male	Female	Male	Male	Male	Male	Male	Female	Male	Male
Onset age of diabetes (yr)	27	Around 40	22	39	33	50	31	47	66	54	34	57
BMI (kg/m^2)	20.24	23.5	21.7	23.9	21	24.2	/	24.54	24.21	28.94	23	23.1
HbA1c (%)	9.1	/	7.6	6.8	7.7	9.8	/	5.6	7.6	7.7	10.9	7.2
FBG (mmol/L)	6.8	/	9.3	7.8	8.3	9.6	16	5.65	8.98	9.44	5.53	8.34
PBG (mmol/L)	21.8	/	11.9	12.7	15.2	17.8	/	5.02	18.82	19.99	17.69	16.85
FINS (pmol/L)	/	/	51.54	61.30	57.11	84.28	/	48.84	26.52	277.56	56.04	85.8
PINS (pmol/L)	/	/	206.16	190.84	134.42	314.12	/	507.3	52.62	562.62	121.38	478.26
FCP (pmol/L)	135.4	/	/	/	/	/	/	/	/	/	/	/
PCP (pmol/L)	600.1	/	/	/	/	/	/	/	/	/	/	/
GADA	+	/	-	-	-	-	-	-	-	-	-	-
IA-2A	/	/	-	-	-	-	-	-	-	-	-	-
Diagnosis	DM	DM	DM	DM	DM	DM	DM	IGT	DM	DM	DM	DM
Complications	DKD, DPN, macroangiopathy	None	/	/	/	/	/	/	/	/	/	/
Therapy	OHA + Insulin	Insulin	OHA→Insulin	OHA	OHA	OHA	OHA	-	OHA	-	OHA	OHA

BMI: Body mass index; HbA1c: Hemoglobin A1c; FBG: Fasting blood glucose; PBG: 2h postprandial blood glucose; FINS: Fasting insulin; PINS: 2h postprandial insulin; FCP: Fasting C-peptide; PCP: 2h postprandial C-peptide; GADA: Glutamic acid decarboxylase antibody; IA-2A: Islet antigen 2 antibody; DM: Diabetes mellitus; DKD: Diabetic kidney disease; DPN: Diabetic peripheral neuropathy; OHA: Oral hypoglycemic agent; IGT: Impaired glucose tolerance.

the proband was positive for islet antibodies.

A2T refers to the substitution of alanine by threonine in the signal peptide, which causes a change in protein secondary structure (α-helix to β-sheet)[7]. Such conformational changes may affect the cleavage of preproinsulin, which is subsequently retained in the endoplasmic reticulum, resulting in endoplasmic reticulum stress, and eventually leads to reduced production of insulin[7].

Apart from this report, there have been two articles consisting of 10 participants that have presented with clinical characteristics for A2T mutation carriers (Table 3)[6,7]. Combined with our research, we found that the A2T mutation does not always result in diabetes mellitus, as evidenced by Yan *et al*[6] study, which found that one person had impaired

glucose tolerance. Diabetic patients who carry A2T mutations typically experience a later onset of diabetes, have a normal BMI, and no islet antibodies. Most patients maintain stable blood glucose levels by using oral drugs. A minority of patients are medicine-free and insulin-independent, but some may require a high dose of insulin, as was the case with the proband and her sister in our study.

CONCLUSION

Herein, we offer a comprehensive summary of the clinical characteristics observed in individuals with MODY10 carrying A2T mutations. Furthermore, we present an atypical MODY10 case resulting from the A2T mutation. The patient exhibited positive islet autoantibodies, as well as demonstrated significant familial inheritance and hearing impairment, which increased the potential for misdiagnosis. We stress that not all patients adhere to the conventional presentation, highlighting the importance of increased vigilance and careful consideration to prevent cases from being overlooked or misdiagnosed.

FOOTNOTES

Author contributions: Chen H was in contact with the patient and wrote the manuscript; Fei SJ, Chen XD, and Wang WH edited specific sections of the manuscript; Deng MQ, Guo LX, and Pan Q reviewed the literature; all authors have read and approved the final manuscript; all listed authors meet the requirements for authorship.

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