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ABOUT COVER

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AIMS AND SCOPE

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WJD mainly publishes articles reporting research results and findings obtained in the field of diabetes and covering a wide range of topics including risk factors for diabetes, diabetes complications, experimental diabetes mellitus, type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, diabetic angiopathies, diabetic cardiomyopathies, diabetic coma, diabetic ketoacidosis, diabetic nephropathies, diabetic neuropathies, Donohue syndrome, fetal macrosomia, and prediabetic state.

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EDITORIAL

Unlocking new potential of clinical diagnosis with artificial intelligence: Finding new patterns of clinical and lab data

Pradeep Kumar Dabla

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Abstract

Recent advancements in science and technology, coupled with the proliferation of data, have also urged laboratory medicine to integrate with the era of artificial intelligence (AI) and machine learning (ML). In the current practices of evidence-based medicine, the laboratory tests analysing disease patterns through the association rule mining (ARM) have emerged as a modern tool for the risk assessment and the disease stratification, with the potential to reduce cardio-vascular disease (CVD) mortality. CVDs are the well recognised leading global cause of mortality with the higher fatality rates in the Indian population due to associated factors like hypertension, diabetes, and lifestyle choices. AI-driven algorithms have offered deep insights in this field while addressing various challenges such as healthcare systems grappling with the physician shortages. Personalized medicine, well driven by the big data necessitates the integration of ML techniques and high-quality electronic health records to direct the meaningful outcome. These technological advancements enhance the computational analyses for both research and clinical practice. ARM plays a pivotal role by uncovering meaningful relationships within databases, aiding in patient survival prediction and risk factor identification. AI potential in laboratory medicine is vast and it must be cautiously integrated while considering potential ethical, legal, and privacy concerns. Thus, an AI ethics framework is essential to guide its responsible use. Aligning AI algorithms with existing lab practices, promoting education among healthcare professionals, and fostering careful integration into clinical settings are imperative for harnessing the benefits of this transformative technology.

Key Words: Laboratory medicine; Artificial intelligence; Machine learning; Association rule mining; Cardiovascular diseases

Core Tip: The integration of artificial intelligence (AI) and machine learning in laboratory medicine presents a promising opportunity to improve the patient care, particularly in the context of multi-factorial cardiovascular diseases. However, it is essential to approach this transformation carefully, side by side addressing ethical considerations, biases, while ensuring its responsible implementation through the collaboration between the technology experts and the healthcare professionals. Education and training are key to unlocking the full potential of AI while safeguarding patient privacy and data.

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INTRODUCTION

Recent developments with advancements of science and technology and production of massive data have helped laboratory medicine to reach the era of artificial intelligence (AI) and machine learning (ML). In the era of evidence-based medicine, combining laboratory testing with associated disease patterns using association rule mining (ARM) can prove to be modern tool for the risk assessment and disease stratification to reduce mortality in cardiovascular diseases (CVD) patients. AI based algorithms have brought more insights and addressed a variety of problems in this field and can be considered as emerging interdisciplinary field[1].

The available literature suggests that the CVDs had occurred earlier in the Indian population as compared to the European population. Further, the fatality rate has found to be even two-fold increase in Indian population in comparison with the same age group. Thus, CVDs have become the leading cause of mortality and source of much needed attention as a global threat. The hypertension, diabetes, metabolic syndrome, smoking, physical inactivity, diet pattern, and other environmental factors were counted as the major responsible factors for the higher rate of CVD in the Indian population [2]. Further, the available data supports the increased mortality with acute coronary syndrome in the young myocardial infarction patients of less than 45 years of age. It is pertinent to note that the CVDs and associated risk in the early stage are typically treated with the greatest probability of success. In another study which is conducted by Dabla *et al*[3], the researchers found the diagnostic edge with the with lipid indices like lipid tetrad index and lipid pentad index to evaluate the atherogenic index of plasma with respect to the higher risk of premature CAD.

Traditionally, physicians diagnose CVDs based on their knowledge from their previous experience with patients with similar clinical presentations. It cannot be ignored that many countries are currently dealing with the shortage of skilled physicians, where AI can prove to be hopeful solution for the overburdened healthcare system. The growing requirement of personalized medicine for modern laboratory practices cannot be denied, resulting in an increasing amount of big data. ML-based techniques and high-quality cleaned data utilising electronic health records (EHRs) presented in the right format, can help to raise the computation analysis, not only for research but for clinical practice as well. The predictive power of computational analysis of EHRs can be enhanced when coupled with imaging and clinical attributes[4]. This unique technique can prove to be a potential tool for the early detection and intervention while applying practical rules to assist doctors and patients in early detection and intervention. There are various methods and rules are applicable in data mining, out of which the ARM technique can extracts potential associations or causal relationships between the sets of patterns present in the given databases[5].

The Advanced Relation Mapping (ARM) method explores the informative index of specified persistent entities or occurrences, establishing connections between elements or events. Consequently, these guidelines unveil noteworthy associations among factors in the data repository, offering a powerful instrument for foreseeing the longevity of individuals experiencing symptoms of cardiac insufficiency. Moreover, it facilitates the identification of crucial clinical attributes (or risk elements) associated with the onset of heart failure. Soni *et al*[6] in 2016 employed an association rule algorithm to assess the potential risks for individuals with diabetes. Their study involved the application of this algorithm to extract relationships within an authentic dataset. Shehabi and Baba[7] in 2021 proposed a novel approach known as Mining Association Rules Classification to extract significant association rules, addressing challenges associated with symbolic methods. This method aims to overcome issues arising from generating an excessive number of association rules in the context of small datasets, a common problem leading to the production of redundant rules in large datasets. In 2022, Singh *et al*[8] employed the hotspot algorithm to identify patterns and associations among various attributes. The analysis encompassed a comprehensive set of biochemical evaluation tests, coupled with a detailed patient history that included physical examinations and electrocardiograms. The biochemical markers measured comprised the lipid profile, encompassing total cholesterol, triglyceride, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, apoprotein A1, apolipoprotein B, and Lp (a) levels. Moreover, it is imperative to acknowledge that the rapid pace of technological evolution and integration demands vigilant consideration of potential medical, ethical, legal, and reputational risks. In this context, ethical considerations are becoming topic of concern and soon necessary requirements. Though, AI application in lab medicine is limited till date compared to other healthcare facilities, however its realization also requires addressing risk of bias tools, algorithm auditing, error managements and most importantly privacy concerns and ethical issues. The significance of an AI ethics framework lies in its ability to illuminate both the potential risks and benefits associated with AI tools, while also setting forth guidelines for their responsible and ethical utilization.

We cannot deny that advantages of new technologies require careful alignment and optimization of AI based algorithms with existing lab practices[9]. Hence, rather than hastily implementing technology, a more prudent approach involves directing its adoption through education and careful integration into clinical practices, ensuring its appropriate use by healthcare professionals.

CONCLUSION

The integration of AI in laboratory medicine holds immense potential to transform healthcare, particularly in combating CVDs. However, its responsible implementation, addressing ethical concerns, and collaboration between technology and healthcare experts are crucial to harnessing the benefits and improve patient outcomes.

FOOTNOTES

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EDITORIAL

Acute worsening of microvascular complications of diabetes mellitus during rapid glycemic control: The pathobiology and therapeutic implications

Dania Blaibel, Cornelius James Fernandez, Joseph M Pappachan

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Abstract

While chronic hyperglycaemia resulting from poorly controlled diabetes mellitus (DM) is a well-known precursor to complications such as diabetic retinopathy, neuropathy (including autonomic neuropathy), and nephropathy, a paradoxical intensification of these complications can rarely occur with aggressive glycemic management resulting in a rapid reduction of glycated haemoglobin. Although, acute onset or worsening of retinopathy and treatment induced neuropathy of diabetes are more common among these complications, rarely other problems such as albuminuria, diabetic kidney disease, Charcot's neuroarthropathy, gastroparesis, and urinary bladder dysfunction are also encountered. The *World Journal of Diabetes* recently published a rare case of all these complications, occurring in a young type 1 diabetic female intensely managed during pregnancy, as a case report by Huret *et al*. It is essential to have a comprehensive understanding of the pathobiology, prevalence, predisposing factors, and management strategies for acute onset, or worsening of microvascular complications when rapid glycemic control is achieved, which serves to alleviate patient morbidity, enhance disease management compliance, and possibly to avoid medico-legal issues around this rare clinical problem. This editorial delves into the dynamics surrounding the acute exacerbation of microvascular complications in poorly controlled DM

during rapid glycaemic control.

Key Words: Diabetes mellitus; Microvascular complications; Diabetic retinopathy; Treatment induced neuropathy of diabetes; Diabetic nephropathy; Charcot's neuropathy

Core Tip: New onset, or acute worsening of preexisting microvascular complications of diabetes mellitus (DM), is an uncommon complication of rapid improvement of chronic hyperglycaemia from intensive management of DM. Worsening of diabetic retinopathy and treatment induced neuropathy of diabetes are the two common microvascular diseases complicating intensive DM treatment with a rapid glycated haemoglobin reduction more than 2% points within a period of 3 months, though less commonly other complications such as Charcot's neuroarthropathy, diabetic nephropathy, gastroparesis and urinary bladder dysfunction are also encountered. This editorial discusses the case of a young female type 1 diabetic, intensively managed during pregnancy, developing all these complications, published as a case report in the *World Journal of Diabetes*, with an appraisal of the current evidence on this uncommon phenomenon.

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INTRODUCTION

Uncontrolled chronic hyperglycaemia is associated with both micro- and macrovascular complications. The microvascular complications including diabetic nephropathy (DN), retinopathy, and neuropathy could lead to dialysis, blindness, and amputation, respectively[1,2]. On the other hand, macrovascular complications including myocardial infarction, heart failure, and stroke could increase the mortality risk[3]. Studies in type 2 diabetes mellitus (T2DM) patients have shown that certain microvascular complications are associated with increased risk for macrovascular complications and cardiovascular mortality[4]. Though intensive control of chronic hyperglycaemia could reduce the incidence of microvascular (statistically significant) and macrovascular (statistically not significant) complications[5], the global burden of these complications is still alarmingly high due to the increased prevalence of diabetes mellitus and increased life expectancy[6].

However, rapid control of chronic hyperglycaemia in those with longstanding poorly controlled diabetes can be associated with worsening microvascular complications including diabetic retinopathy (DR)[7,8], painful diabetic neuropathy (PDN)[9,10], Charcot's neuroarthropathy (CN)[11-14], and rarely DN[15]. This paradoxical worsening of diabetic microvascular disease can occur in patients with both type 1 diabetes mellitus (T1DM) and T2DM, with insulin and non-insulin-based therapies, during pregnancy, post-bariatric surgery, and post-pancreas transplant[16].

EARLY WORSENING OF DR

The diabetes control and complications trial (DCCT) trial observed an early worsening of DR among T1DM patients with high glycated haemoglobin (HbA1c) levels at baseline and a marked HbA1c reduction in the initial 6 months of treatment [17]. Despite this early worsening, the intensively treated T1DM group had an equivalent or superior long-term retinopathy outcome in comparison to the conventionally treated group without early worsening. Coexistent renal impairment, presence of advanced DR (severe non-proliferative DR or proliferative DR), younger age at T1DM diagnosis, raised serum triglyceride levels, and increased retinal venular diameters are the known predictors for excessive risk of DR progression in those patients achieving rapid glycaemic control[17,18].

Pathogenesis of early worsening of DR

There are several studies in the literature that describe early worsening of DR with tighter glycaemic control[7,8,16-18]. For instance, in a study conducted at Oslo, half of the T1DM patients managed with continuous subcutaneous insulin infusion (CSII) experienced early worsening of DR within three months of intense glycemic control by the treatment, as opposed to those on short or intermediate-acting insulin regime[7,19]. Furthermore, similar findings were described in the Kroc Collaboration study, in which 47% of T1DM patients receiving CSII developed worsening DR at 8 months, while only 27% in the conventional treatment group had DR worsening[20]. The mechanism underlying the early worsening of retinopathy is not well established, however, there are several proposed modes of pathogenesis. The clinical or morphological early worsening of DR is defined as the new development of dot-and-blot haemorrhages, microaneurysms, intraretinal microvascular abnormalities (IRMAs), cotton-wool spots (also known as soft exudates), and capillary-free

areas[7]. For example, in the aforementioned Kroc Collaboration study, higher numbers of cotton wool spots and IRMAs were observed in the intensely treated CSII group compared to the conventionally treated group at the 8 months of treatment[7,20].

However, it is noteworthy that at the end of 2 years of treatment, both groups exhibited a similar number of cotton wool spots and IRMAs indicating probable arrest of progression of disease after an initial worsening of DR. There are also some discrepancies in the observed DR outcomes between different studies comparing intense *vs* conventional glycemic control. In the Oslo study for instance, the CSII group had a significantly higher number of microaneurysms compared to the control group at three months of treatment while this group had considerably fewer microaneurysms at baseline (prior to the study)[19]. Similar findings were reported in DCCT trial in which an early DR worsening was observed in the intensively treated group with a rapid reduction of HbA1c at 6 months of treatment[17].

It is suggested that two factors contribute to early worsening DR, namely a glycaemia-related mechanism as well as a potential role for blood pressure (BP) control. A rapid drop of HbA1c with intensive glycaemic treatment lowers intravascular osmotic pressure[21]. The resultant osmotic gradient between intra- and extracellular compartments causes water movement across blood vessels with fluid exudation from the vulnerable retinal microcirculation[7]. Another proposed mechanism is a possible synergistic effect of insulin and vascular endothelial growth factor (VEGF) on retinal vessels with proliferation of retinal vessels and worsening of DR[22]. This overexpression of VEGF coupled with the release of reactive oxygen species in the context of hyperglycaemia might contribute to worsening of DR. Tight BP control also might contribute to worsening DR though this mechanism remains controversial with contrasting evidence[7].

EARLY WORSENING OF PDN (TREATMENT INDUCED NEUROPATHY OF DIABETES)

The commonest microvascular complication associated with intensive glycaemic control is Treatment Induced Neuropathy of Diabetes (TIND), previously also known as insulin neuritis, characterised by painful sensory neuropathy and at times, autonomic neuropathy. TIND is defined as the occurrence of an acute neuropathy within 8 wk of a rapid decrease in HbA1c often following rapid improvement of a chronic hyperglycaemic state[10]. The actual prevalence of TIND is unknown but may be encountered in up to 10% of tertiary referrals for evaluation of acute diabetic neuropathies [9]. Although TIND is more commonly seen in individuals with T1DM, it may also occur in T2DM managed with hypoglycaemic medications or even diet control[9].

Pathogenesis of TIND

Although the exact mechanisms are not fully understood, experiments in animal models suggested various concepts. One is the hypothesis of neural "energy crisis" wherein, chronic hyperglycaemia might lead to a reduction in nerve blood flow and endoneural oedema, creating a long-standing hypoxic endoneural microenvironment[10]. When an abrupt drop in glucose levels occurs in such as situation, it might lead to a relative endoneural hypoglycaemia, leading to an "energy crisis", resulting in acute neuropathy.

Another factor is the presence of macrophage infiltration in peripheral nerves leading to chronic inflammation and the release of macrophage-derived cytokines and neuroinflammatory regulators which result in the activation of sensory neurons resulting in neuropathic pain[10]. Neural regeneration after improved glycemic control also can be an additional source of neuropathic pain[23]. However, this concept requires further studies for a comprehensive understanding of how the interplay between nerve degeneration and regeneration contributes to TIND.

ACUTE DEVELOPMENT/ WORSENING OF CN

CN is a chronic destructive disease in patients with peripheral neuropathy characterized by painful or painless bone and joint destruction in limbs that have lost sensory innervation[16]. The estimated prevalence of CN is 0.1% to 0.4% in individuals with diabetes, but the prevalence can be as high as 35% in patients with diabetic peripheral neuropathy[16]. Both types of diabetes can increase the risk, though the prevalence may be higher in those with T1DM and one or both feet may be involved.

Pathogenesis of acute Charcot's following rapid improvement of diabetes

Bone modelling factors such as RANKL (receptor activator of nuclear factor-κB ligand) and its antagonist osteoprotegerin (OPG) play an important role in the development of CN[16]. Acute CN may occur with rapid correction of hyperglycaemia as in patients who underwent a concurrent kidney–pancreas transplantation. This is possibly from an interplay of OPG and RANKL levels resulting from intensive glycemic control in such situations[16]. Inhibition of OPG by reduction of HbA1c might induce an increase in the RANKL level leading to rapid maturation of the osteoblasts, and hence bone lysis with the development of acute CN.

ACUTE WORSENING OF DN WITH RAPID GLYCEMIC CONTROL

Like the observed development of DR and TIND with the rapid reduction of HbA1c, there is evidence, however, limited, in the literature that indicates a rapid correction of hyperglycaemia may contribute to the early worsening of DN. For

instance, in an article by Cundy *et al*, the authors describe a rapid and sustainable decline of eGFR (estimated glomerular filtration rate) in four subjects with type 2 diabetes after their HbA1c had fallen from 118 mmol/mol (12.9%) to 48 mmol/mol (5.5%)[15]. The renal microcirculation is usually protected by hemodynamic autoregulation when compared to the retina and the vasa nervorum of peripheral nerves which would explain the rarity of this phenomenon.

TIND AND DIABETIC AUTONOMIC NEUROPATHY

There is a strong correlation between the proportionate risk of development of TIND and the magnitude of HbA1c reduction as observed by a large series by Gibbon and Freeman[24]. For instance, a rapid HbA1c reduction of 2%-3% points in 3 months period was shown to be associated with an absolute risk of TIND in 20% of diabetic subjects while a reduction of > 4% was associated with an absolute risk of > 80% in this study. Complications of diabetic autonomic neuropathy such as gastroparesis, urinary bladder dysfunction, erectile dysfunction, and cardiac autonomic neuropathy are all consequences of TIND[24]. The Figure 1 shows the microvascular complications that can be associated with rapid improvement of chronic hyperglycaemia in patients with poorly controlled diabetes.

SIMULTANEOUS DEVELOPMENT OF MULTIPLE MICROVASCULAR COMPLICATIONS

Although there are several cohort studies, case series and reports in the published literature, the simultaneous occurrence of a multitude of microvascular diabetic complications following rapid correction of severe chronic hyperglycaemia is rarely reported. Huret *et al*[25] in the recent issue of the *World Journal Diabetes*, reported a unique case of multifocal microvascular disease complicating the management of a 25-year-old woman with poorly controlled T1DM after intense reduction of chronic hyperglycaemia during pregnancy. The patient developed crippling CN (culminating in left knee replacement and foot amputation) followed by the development of severe sight-threatening DR, gastroparesis, urinary bladder dysfunction, and rapidly progressive DN terminating in dialysis dependence within three years of the index disease onset.

Although the development of microvascular complications because of rapid reduction of chronic hyperglycaemia occurs usually within a short period of time, the multifocal microvascular disease in the above case except CN occurred only after several months of marked improvement of her T1DM, which is slightly difficult to explain. We are unclear whether pregnancy as such impacted the delay in the development of some of these complications, in this case, owing to the marked immune alterations at the childbearing period in females. It is noteworthy that some of the microvascular complications are believed to develop through immune/cytokine-mediated mechanisms, and the immunological and cytokine milieu during pregnancy are very different from those in normal adults.

Pregnancy as such is a risk factor for the development and/or progression of microvascular complications in patients with preexisting diabetes possibly because of marked glycaemic fluctuations during this period[26], for which reason, women are regularly monitored during each trimester of their pregnancy for screening these complications. Although rigorous control of hyperglycaemia was very important during pregnancy to improve maternal and foetal outcomes, this patient, unfortunately, developed catastrophic complications of intense diabetes control, an unanticipated and possibly unavoidable iatrogenic complication.

MEDICOLEGAL IMPLICATIONS OF THE DEVELOPMENT OF MICROVASCULAR DISEASE WITH MARKED GLYCEMIC CONTROL

A few potential medicolegal issues may arise in relation to an acute worsening of microvascular complications of diabetes from tight glycaemic control. For instance, there is an issue with informed decision making, and establishing a standard of care. Physicians and healthcare providers should ensure that patients are adequately informed about the potential risks associated with tight glycaemic control and the possibility of early worsening of microvascular complications. Informed consent should include discussions about the benefits and risks of treatment options, potential complications, and alternatives. Failing to obtain informed consent may lead to legal challenges if complications arise.

Moreover, healthcare providers are expected to adhere to the standard of care when managing diabetes and its complications. If a provider fails to follow established guidelines and recommendations, it may be considered medical malpractice. However, it is critical to note that there are no current large-scale studies that examined the cut-off points for HbA1c that is optimal for evading these complications simultaneously, while aiming to curb the long-term effects of poor glycaemic control.

Situations, where marked reduction of hyperglycaemia and HbA1c are anticipated, as in cases of bariatric surgery, pregnancy (as in the case described by Huret *et al*[25]), pancreas transplantation, and even in those with massive weight loss from lifestyle changes, incretin-based therapies, or bariatric surgery, patients may be counselled well in advance regarding likelihood of development of these microvascular complications. Patients also must be monitored rigorously and regularly in such situations.

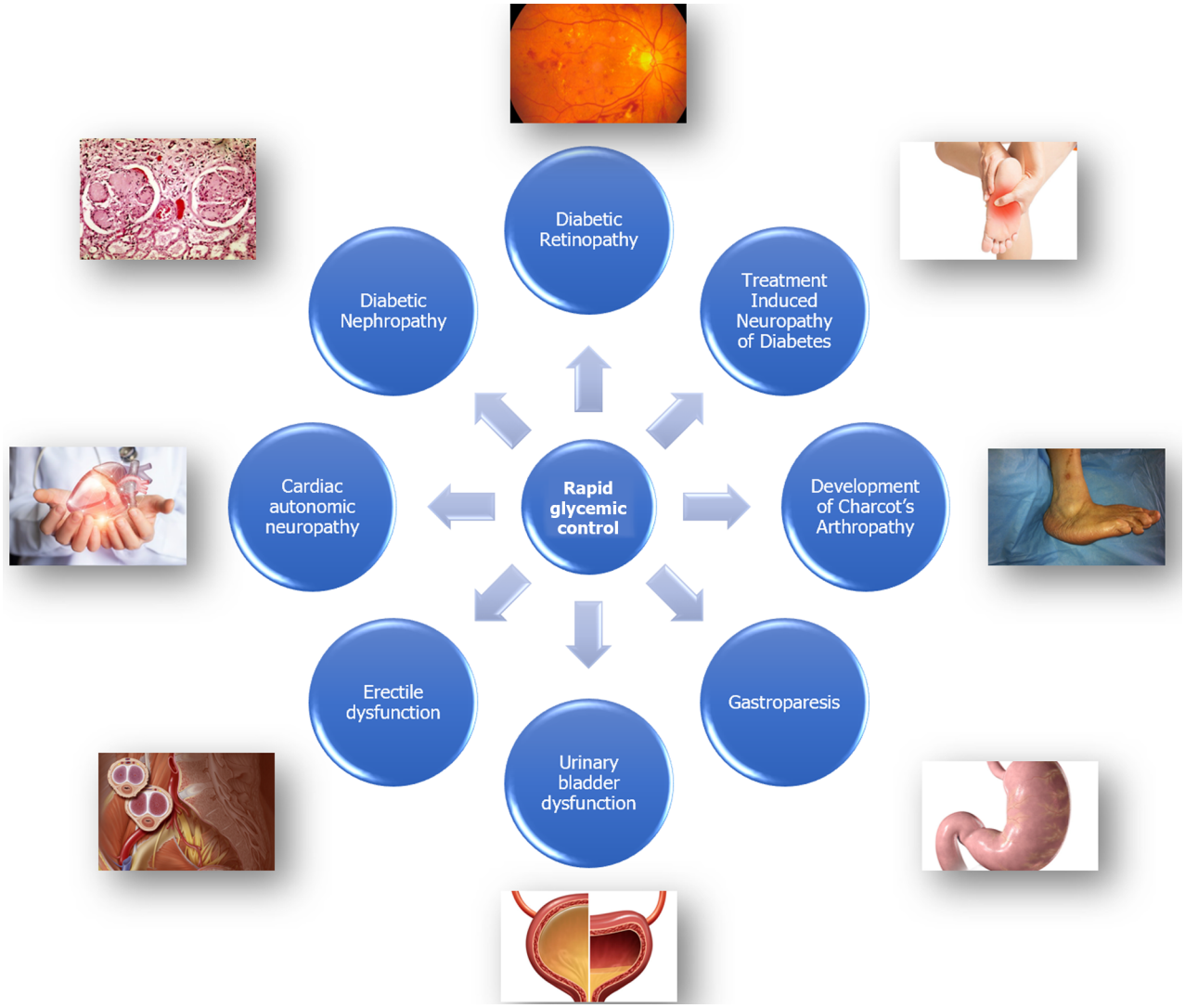

Figure 1 Probable acute worsening of microvascular complications that can develop following rapid and marked improvement of hyperglycaemia in patients with poorly controlled diabetes mellitus.

THERAPEUTIC STRATEGIES

Optimal management strategy of TIND is still not clear because of the lack of adequate data from global scientific literature. Some experts suggest relaxing the glycaemic targets transiently to slow down the intensity of rigorous control of hyperglycaemia, while others do not support this concept and advise supportive management[27-29]. There is some evidence favouring the concept of relaxing the tight glycaemic control (permissive hyperglycaemia) for short periods in patients developing acute DR/worsening DR complicating the abrupt diabetes improvement[27,30]. However, maintaining good glycaemic control over long-term periods with only symptomatic supportive management of TIND has resulted in improvement of the disease in a good proportion of patients, while marked fluctuations and poor glucose control have been associated with worsening of all the microvascular complications including DR, TIND, and nephropathy[30]. Therefore, it is better to target an HbA1c reduction of < 2% points within the first three months of glycaemic management, if possible, in patients with poor baseline glycaemic control.

As there is inadequate data on the appropriate treatment of acute onset/worsening of existing microvascular complications in patients with poor baseline diabetes control, a patient-cantered management strategy must be adopted depending on the individual clinical situation. Wherever an abrupt reduction of HbA1c of more than 2% points is expected (*e.g.*, bariatric surgery, marked weight loss in obesity with T2DM, and in eating disorders), patients should be counselled and regularly monitored. Management options as mentioned above may be considered, though improvement may not occur in some patients as reported by Huret *et al*[25].

CONCLUSION

Acute worsening of microvascular complications is a challenging aspect of managing uncontrolled diabetes. Uncontrolled

chronic hyperglycaemia is a well-established risk factor for both microvascular and macrovascular complications, leading to significant morbidity and mortality. However, the paradoxical exacerbation of microvascular complications, such as DR, PDN, CN, and DN, during rapid glycaemic control can be an important clinical concern with probable medicolegal implications. The pathobiology, actuarial prevalence, predisposing factors, and the management strategies for acute onset/ worsening of microvascular complications in patients achieving rapid glycaemic control of their poorly controlled diabetes are yet to be fully elucidated. More research with emerging new evidence on this enigmatic disease may help us to optimally manage patients like the one reported by Huret *et al* in their unique clinical case report.

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EDITORIAL

Periodontitis: An often-neglected complication of diabetes

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Abstract

The bidirectional association between type 2 diabetes mellitus (T2DM) and periodontitis is now well established, resulting in periodontal disease being considered as the 6th major complication of diabetes mellitus (DM) after cardiovascular disease, eye disease, neuropathy, nephropathy, and peripheral vascular disease. DM can worsen the virulence and invasiveness of pathogenic oral microbial flora aggravating the local inflammation and infection in those with periodontal disease. On the other hand, the chemical and immunological mediators released into the circulation as part of periodontal inflammation worsen the systemic insulin resistance with worsening of T2DM. Periodontitis if undiagnosed or left untreated can also result in eventual tooth loss. A study by Xu *et al* in the *World Journal of Diabetes* examined the predictive factors associated with periodontitis in Chinese patients with T2DM. The prevalence of periodontitis was found to be 75.7% in this study. Based on logistic regression analysis, the predictive factors for higher risk were low tooth brushing frequency [odds ratio (OR) = 4.3], high triglycerides (TG; OR = 3.31), high total cholesterol (TC; OR = 2.87), higher glycated hemoglobin (HbA1c; OR = 2.55), and higher age (OR = 1.05) while higher education level was protective (OR = 0.53). However, the most influential variables were HbA1c followed by age, TC, TG, low education level, brushing frequency, and sex on the random forest model (this model showed higher sensitivity for predicting the risk). A good understanding of the predictors for periodontitis in T2DM patients is important in prevention, early detection of susceptible patients, and intervention to improve periodontal health and enable long-term glycaemic control as observed by Xu *et al*.

Key Words: Diabetes mellitus; Periodontitis; Predictive factors; Cardiovascular disease; Glycaemic control

Core Tip: The bidirectional association between type 2 diabetes mellitus (T2DM) and periodontitis is now well established, resulting in periodontal disease being considered as the 6th major complication of diabetes mellitus (DM) after cardiovascular disease, eye disease, neuropathy, nephropathy, and peripheral vascular disease. Periodontal inflammation worsens systemic insulin resistance with worsening of DM. A higher prevalence of periodontitis is seen in patients with poor glycemic control presumably from increased level of inflammation and risk of tissue destruction in these patients. Periodontitis if undiagnosed or left untreated can result in eventual tooth loss. A study by Xu *et al* in the recent issue of the *World Journal of Diabetes* highlights the predictive factors associated with periodontitis in patients with T2DM to enable readers to have a better understanding of both diseases.

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INTRODUCTION

Gingival and dental health are very sensitive markers of systemic health and well-being, though healthcare providers often underrecognize their importance largely because of inadequate awareness about this interlink. The dental and gingival disease can result in the worsening of several systemic illnesses including diabetes mellitus (DM), and DM can aggravate oral ill-health in a vicious circle[1]. The oral cavity is the habitat for billions of microorganisms, and changes in this biological ecosystem may be associated with several local and systemic disorders that can cause serious human health issues.

Periodontitis is a chronic inflammatory disorder affecting supportive tissues of teeth which results in tooth loss unless appropriate management is instituted on time[2]. The disease severity may range from mild illness without serious consequences to extreme cases with tooth loss as an unavoidable complication. Periodontitis affects more than 50% of the adult global population with an estimated 1.1 billion severe cases in the year 2019, and a 68% increase in the prevalence of severe disease from 1990 to 2019[3]. Apart from local complications, periodontitis can also be associated with several systemic diseases such as cardiovascular disease (CVD), lung disorders, chronic kidney disease (CKD), cirrhosis, obesity, colitis, rheumatoid arthritis, osteoporosis, malignancies, type 2 DM (T2DM), and even Alzheimer's disease[4,5]. Although the exact mechanisms interlinking these systemic diseases and periodontitis are not fully elucidated, population-based studies reveal clear associations. Figure 1 shows the association between periodontitis and various systemic diseases[5].

PATHOPHYSIOLOGY OF PERIODONTITIS AND SYSTEMIC DISEASES

Periodontitis-related inflammation of gum tissues and the host response to microbial dysbiosis results in the gradual destruction of the dental supporting structures with loosening of the tooth and expulsion over time. This inflammation also evokes the release of several immune and inflammatory cytokines and other chemical factors into the circulation which might in turn initiate or perpetuate the worsening of several systemic diseases.

Periodontitis and CVD

A recent systematic review suggested a 7.2% prevalence of CVD and its significant association in men (OR = 1.22) and women (OR = 1.11) with periodontal disease[6]. The highly vascular nature of gums and periodontal issues result in the dissemination of bacteria and their toxins into circulation regularly. These initiate and potentiate systemic inflammatory responses and acceleration of atherosclerotic process as a direct consequence of endotoxemia and indirectly through various cytokines like C-reactive protein (CRP), interleukin-6, tumor necrosis factor-alpha and interferon-gamma[7,8]. Atherosclerosis is the main cause of most of the CVD subtypes such as hypertension, coronary heart disease, heart failure, and stroke.

Pulmonary disease and periodontitis

The strong interlink between lung disease and periodontitis was reported by a recent systematic review which found statistically significant positive associations with chronic obstructive pulmonary disease (COPD) and obstructive sleep apnea, while few studies revealed positive benefits of treatment of periodontitis in those with COPD, asthma, and bacterial pneumonia[9]. Micro-aspiration of oral microbes and systemic immune mechanisms related to oral inflammation impacting a harmful immune response in the respiratory system might explain these interlinks[5]. Moreover, oral

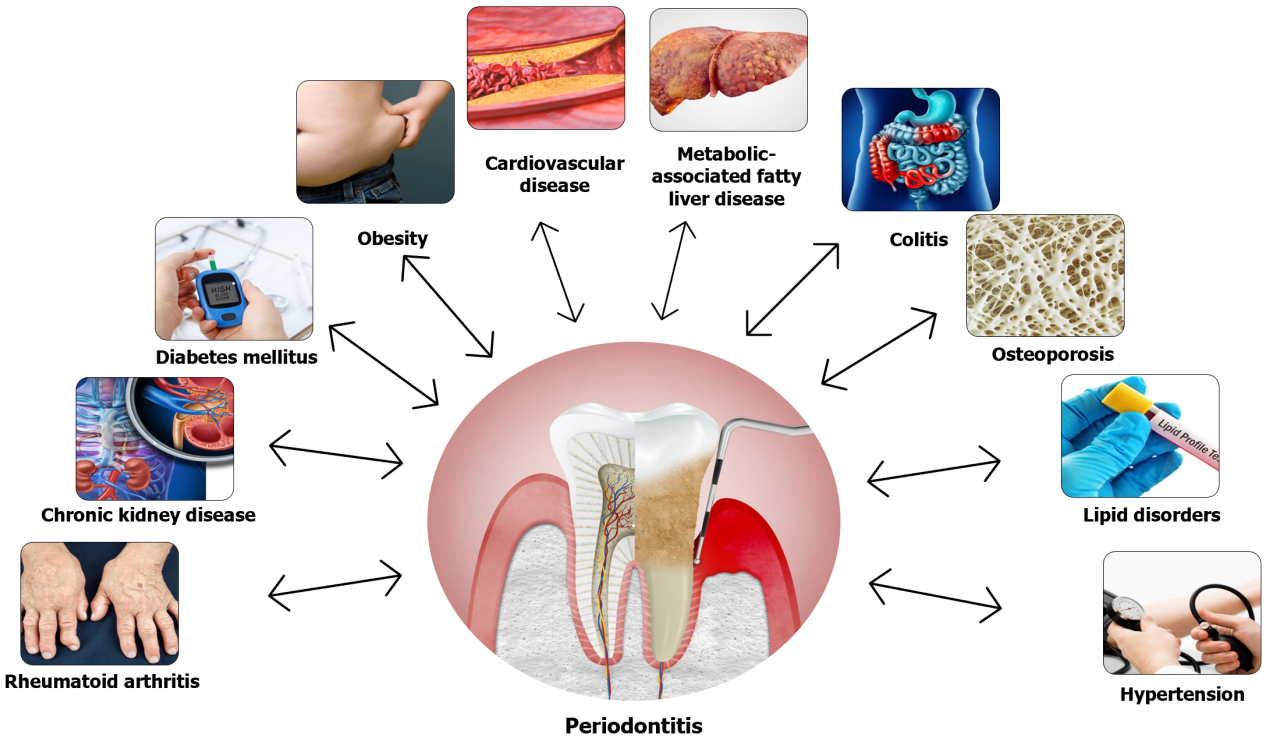

Figure 1 The association between periodontitis and systemic diseases.

infection with systemic bacteremia can sometimes result in pulmonary infections and even a lung abscess.

Periodontitis and CKD

A recent meta-analysis showed a significant association (OR: 2.36) between CKD and periodontitis[10]. Another meta-analysis revealed improvement in glomerular filtration rate, markers of inflammation, and possibly even mortality rate in those patients receiving periodontal treatment[11]. These study results emphasize the importance of appropriate dental care for patients with CKD from the therapeutic and prevention perspectives.

Periodontitis and other systemic diseases

Similar humoral and immune mechanisms are involved in the aetio-pathogenesis of other diseases such as cirrhosis, obesity, colitis, rheumatoid arthritis, osteoporosis, malignancies, T2DM, and even Alzheimer's disease[5,12]. Inflammation of periodontal tissues from the local oral microbiome activates lymphocytes with the release of several immune factors into the circulation which results in initiation or aggravation of the systemic comorbidities. On the other hand, these systemic disorders such as DM aggravate the periodontal disease in a vicious circle potentiating both the local and systemic disease[12]. Therefore, clinicians should be vigilant to address both issues in patients they care for. Table 1 summarises the pathobiological interlink between periodontitis and various systemic disorders[5-12].

PATHOBIOLOGICAL INTERLINK BETWEEN PERIODONTITIS AND DM

Since 1993, severe periodontal disease has been considered the 6th major complication of DM after CVD, eye disease, neuropathy, nephropathy, and peripheral vascular disease[13]. Being a state of immunocompromise, DM can worsen the virulence and invasiveness of pathogenic oral microbial flora aggravating the local inflammation and infection in those with periodontal disease[1,14]. Moreover, hyperglycemia-induced oxidative stress and the generation of reactive oxygen species in the periodontal tissues can aggravate local tissue damage and worsen periodontitis[14]. On the other hand, the chemical and immunological mediators released into the circulation as part of periodontal inflammation worsen systemic insulin resistance (IR) with worsening of DM.

Interlink between T2DM and periodontitis

Metabolic syndrome (MetS) and IR are the pathophysiological hallmarks of the disease in most patients with T2DM. The inflammatory process in periodontal tissues may release several cytokines into the systemic circulation along with bacterial toxins potentially aggravating the IR. There is also clear evidence for a significant association between MetS and periodontitis as evidenced by a very recent systematic review examining the interlink[15]. This meta-analysis, including 24567 participants from 14 studies, showed that the adjusted odds ratio (OR) for MetS among patients with moderate periodontitis was 1.26 and those with severe periodontitis was 1.50.

Table 1 The pathobiological interlink between periodontitis and various systemic disorders: The potential mechanisms

Systemic disease	Interlink between periodontitis and the disease	Potential mechanisms
CVD	Accelerated atherosclerosis resulting in ischemic heart disease, stroke, peripheral vascular disease, and heart failure	Aggravation of dyslipidemia, insulin resistance, and endothelial dysfunction from release of various toxic cytokines and microbial products
Pulmonary disorders	Pneumonia caused by systemic spread of pathogens from periodontal tissues; Exacerbation of asthma and COPD from spread of pathogens from mouth; Sleep apnoea with high cytokine load worsens periodontitis	Direct and indirect results of oral microbes on pulmonary diseases and systemic cytokines worsening periodontal inflammation
CKD	CKD can worsen periodontitis and vice versa	Immunological and inflammatory responses from either disease
MAFLD & cirrhosis	Periodontitis can increase insulin resistance and advanced liver disease can worsen periodontal inflammation	Inflammatory mediators such as cytokines and chemokines aggravate either disorder
Obesity	Unhealthy lifestyles lead to obesity and periodontitis	Both diseases have inflammatory pathogenesis and can perpetuate each other
Colitis	Oral and gut microbiota are closely interlinked and can perpetuate disease processes in either region	Inflammatory and immune alterations in gut and periodontal tissues can aggravate both diseases
Rheumatoid arthritis	Inflammation of joints and oral tissues can modulate both diseases	Various humoral and immunological factors and cytokines worsen diseases
Osteoporosis	Pathogenesis of systemic and alveolar bone loss are closely linked	Inflammatory processes in periodontal tissues can potentially impact bone loss
Malignancies	Inflammation in periodontal areas can increase risk of malignancy	Various immunological and inflammatory factors from periodontitis can worsen the risk of cancer
Alzheimer's disease	Periodontal disease can worsen Alzheimer's disease and dementia can worsen mouth care and worsen periodontal disease	Increased deposition of β-amyloid and hyperphosphorylation of Tau protein is seen in patients with periodontal disease
Diabetes	Both T1DM and T2DM can worsen periodontitis and periodontitis can worsen glycaemic control	Aggravation of insulin resistance in periodontal disease and poor glycaemic control can worsen oral infections

CVD: Cardiovascular disease; CKD: Chronic kidney disease; COPD: Chronic obstructive pulmonary disease; MAFLD: Metabolic-associated fatty liver disease, T1DM: Type 1 diabetes mellitus, T2DM: Type 2 diabetes mellitus.

Type 1 DM and periodontitis

The prevalence of periodontitis among type 1 DM (T1DM) patients was 18.5% higher with an OR of 2.51 compared to the general population[16]. It was found that the markers of severity of periodontitis such as plaque index, bleeding on probing, pocket depth, gingival index, and clinical attachment loss were significantly higher among children and adolescents with T1DM[17]. There is also evidence for a higher prevalence of periodontitis in patients with poor glycemic control presumably from increased level of inflammation and risk of tissue destruction in these patients[18]. Local mechanisms of tissue defense against inflammation may be poor with higher microbial activity among patients with poor control of T1DM making them more vulnerable to severe periodontal disease.

CLINICAL AND DIAGNOSTIC EVALUATION

It is crucial to understand the bidirectional relationship between periodontitis and DM for optimal management of patients in day-to-day clinical practice. A prompt collaboration between the dentist and the diabetologist should enhance patient outcomes when both diseases co-exist. Regular annual screening for micro- and macro-vascular complications of diabetes is meticulously performed by most clinicians. However, a thorough dental examination is often ignored even among patients with poor glycemic control. Although the annual dental review is recommended in adults with DM, a recent large population-based United States study showed that only 60% of patients performed annual dental check-ups [19].

This underscores the importance of increasing the awareness of oral health among diabetics. Primary care physicians, internists, and diabetologists should encourage DM patients to have annual dental evaluations and utilize the opportunity to refer patients for urgent dental reviews when they notice moderate to severe dental disease during their clinic visits for diabetes care.

CLINICAL DENTAL REVIEW

Poor oral hygiene results in plaque biofilm accumulation on the tooth and surrounding gingival tissues resulting in inflammation and progressive damage of supporting periodontal tissues and bone in susceptible patients[20]. The surface area of the palm is the approximate size of the surface area of periodontal tissue inflammation and ulceration in a periodontitis patient. Periodontitis if undiagnosed or left untreated can result in bleeding gums, periodontal pocket, clinical attachment loss resulting in recession, furcation involvement, suppuration, tooth mobility, and eventual loss of the affected tooth[21].

DIAGNOSTIC EVALUATION

The revised classification of periodontitis is based on the 2017 World Workshop on the classification of periodontal and peri-implant diseases and conditions[22]. This resulted in defining periodontal health and gingivitis based on whether there was bleeding on probing. Gingival health was considered if there were < 10% bleeding sites and less than or equal to 3 mm of probing depth. Staging of periodontitis into stages I, II, III, or IV was based on clinical attachment loss, depth of periodontal pocket, or both[22]. The classification included systemic diseases like DM as modifying periodontal disease and is now stated in the diagnosis along with the staging and grading of periodontitis[23].

A meticulous clinical examination during the annual dentist review for patients with DM involves a basic periodontal examination (BPE) to initially screen for periodontal disease[24]. A BPE score of 3 or 4 would need further investigation which involves a six-point pocket depth charting, bleeding and plaque scores, mobility, recession, furcation involvement, and appropriate radiographs. It is now well established that if an individual has periodontitis, then it is a life-long diagnosis of the disease despite treatment since regular supportive care is essential to maintain healthy periodontal tissues[24].

The pretreatment and post-treatment charting of periodontal indices helps to assess the periodontal disease progression. These scores are repeated annually for patients who are stable following periodontal treatment.

MANAGEMENT ASPECTS

A Cochrane review update published in 2022 on periodontal management for diabetic patients has reported a moderate level of evidence for improvement in glycaemic control until one year following non-surgical periodontal treatment which involves plaque and calculus removal in subgingival areas by ultrasonic or manual method[25]. There was a significant change in glycaemic control in individuals with diabetes and periodontitis in comparison to untreated individuals or those who received normal treatment. The results from thirty studies included in this review revealed that in patients with DM, there was a 0.43% reduction in the level of glycated hemoglobin (HbA1c; from 7.43% to 7%) after a period of 3 to 4 months following periodontal treatment when compared to patients with routine care or no intervention. This difference was 0.30% after 6 months in 12 trials, whereas one study revealed a 0.5% reduction in HbA1c after 12 months[25].

A well-integrated pathway for medical and dental care of diabetic patients for evaluation of periodontal health and provision of appropriate periodontal treatment is widely recommended[26]. Preliminary sequential management of periodontitis involves patient education involving home care instructions on oral hygiene to enable long-term control of plaque, microbial biofilm, and factors causing periodontal disease, followed by supragingival professional mechanical plaque removal.

In the next stage, subgingival mechanical debridement is done to remove biofilm and calculus deposits using ultrasonic or manual instruments or in combination. The previous terminology ‘scaling and root planning’ is now replaced by ‘subgingival instrumentation’ based on the European Federation for Periodontology S3 treatment guidelines [27].

In advanced cases of periodontitis where the above non-surgical management has failed to show signs of improvement, the next stage of treatment involves surgical management by a periodontist. This involves direct access to subgingival sites of residual disease in patients who have good oral hygiene. Multiple visits may be needed in some cases. However, teeth that do not respond to treatment and have poor prognoses will need extraction to minimize the systemic effects of disease progression and to restore periodontal health[27].

Adjuncts like antiseptic mouthwash like chlorhexidine, and local and systemic antibiotics have been used to treat periodontitis. Currently, there is no clear evidence for the routine use of these antimicrobials along with periodontal therapy[27].

A systematic review and meta-analysis by Cao *et al*[28] looked at no treatment and different modalities of periodontal treatment which included antimicrobial photodynamic therapy, sub-antimicrobial dose of doxycycline, antibiotics (metronidazole, amoxicillin doxycycline), local drug delivery (simvastatin gel, chlorhexidine gel, atorvastatin gel), diode laser and subgingival instrumentation. It was found that a combination of antimicrobial photodynamic therapy, and doxycycline along with subgingival instrumentation was the most effective treatment modality for decreasing HbA1c% in non-smokers with chronic periodontitis who did not have severe complications of T2DM, though the authors of the review acknowledge that the certainty of evidence of this observation was low, or very low.

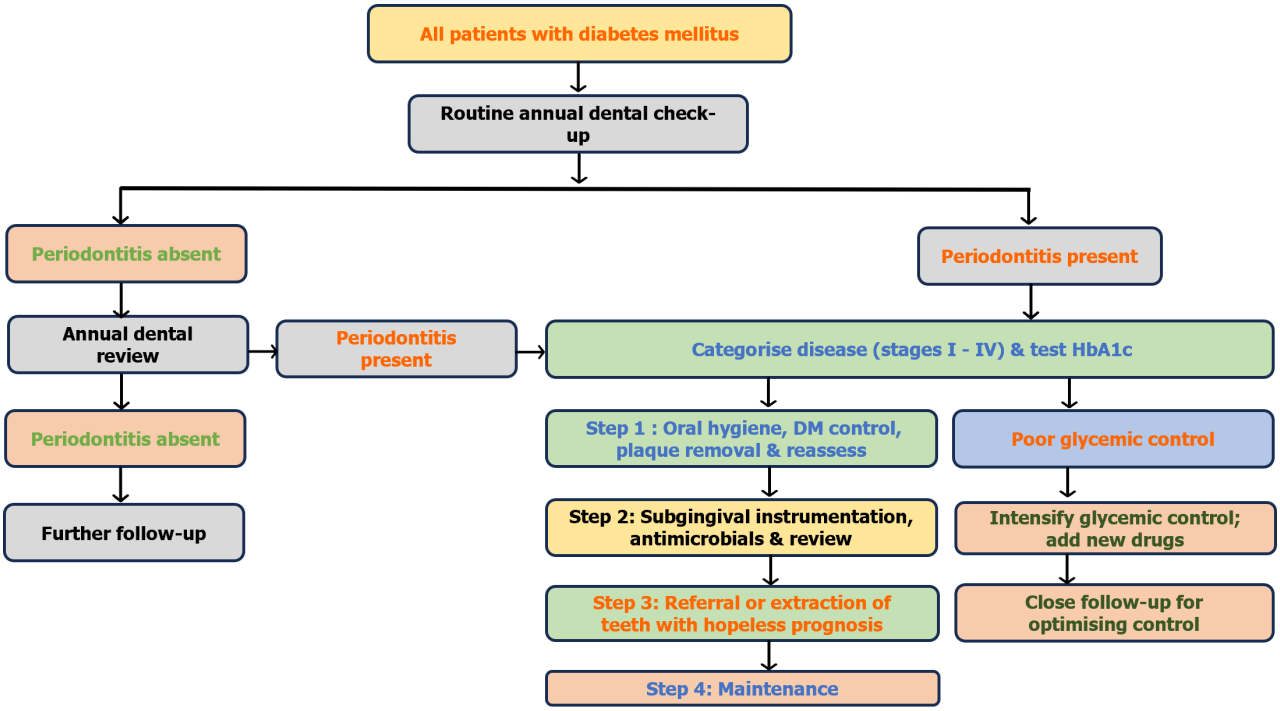

Figure 2 A management algorithm for management of patients with diabetes mellitus and periodontitis. HbA1c: Glycated hemoglobin.

Thus, patient motivation, home care instructions on oral hygiene, professional mechanical plaque removal combined with surgical or non-surgical management, and use of local or systemic antimicrobial drug delivery, and supportive therapy are important for active intervention and long-term maintenance of periodontal health. Figure 2 summarises the management algorithm for patients with diabetes and periodontal disease.

PREDICTING FACTORS FOR PERIODONTITIS IN T2DM

The bidirectional association between T2DM and periodontitis is well established as pointed out by a systematic review by Wu *et al*[29] examining 53 observational studies. The adjusted OR for the prevalence of T2DM in patients with periodontitis was 4.04, while the adjusted OR for the prevalence of periodontitis in patients with T2DM was 1.56 in this study. However, several confounding factors such as the triglyceride level, white blood cell count, CRP, hypertension, waist circumference, body mass index, sex, education, age, income, and dental check-ups might have weakened the evidence level.

As mentioned in the previous section, several factors predispose to the development of periodontitis in patients with T2DM. A study by Xu *et al*[30] in the recent issue of *World Journal of Diabetes*, examined the predictive factors associated with periodontitis in patients with T2DM. The study reported a very high prevalence of periodontitis (75.7%) among Chinese patients. The predictive factors for higher risk were low tooth brushing frequency (OR = 4.3), high triglycerides (OR = 3.31), high cholesterol (OR = 2.87), higher HbA1c (OR = 2.55), and higher age (OR = 1.05) while higher education level was protective (OR = 0.53) on logistic regression analysis, while the most influential variables were HbA1c followed by age, total cholesterol, triglycerides, low education level, brushing frequency, and sex on the random forest model. In the study, the random forest statistical model showed a higher sensitivity for predicting the risk compared to the logistic regression model [area under the curve (AUC) 1.000 *vs* 0.851; $P < 0.05$] in their training dataset, while the validation dataset did not show such a difference (AUC = 0.946 *vs* 0.915; $P > 0.05$). Although other studies looked at the factors associated with periodontitis and T2DM, the study by Xu *et al*[30] provides us reasonably comprehensive evidence for the predictive factors for the disease in the diabetic population. However, we need larger multi-centre prospective cohort studies and randomized controlled trials to generate more robust evidence for clearly proving the definite association between these predictive factors and periodontitis.

CONCLUSION

There is no doubt among scientific professionals regarding the bidirectional association between periodontitis and DM. Periodontal disease is now considered as the 6th major complication of DM after CVD, eye disease, neuropathy, nephropathy, and peripheral vascular disease. Both DM and periodontitis can perpetuate the disease process of each other, and without the appropriate and adequate care, each condition can worsen the other disease to cause significant

health issues among sufferers. Periodontitis is also associated with several systemic diseases which can directly or indirectly worsen the periodontal disease process. Thorough understanding about the pathobiology, clinical presentation and diagnostic work-up of patients with periodontitis and DM is important among healthcare professionals managing these diseases to improve clinical outcomes. The study by Xu *et al*[30] in the Journal examining the predictive factors associated with periodontitis and DM is such an attempt to empower clinicians caring for such patients across the globe.

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EDITORIAL

Glucagon-like-peptide-1 receptor agonists and the management of type 2 diabetes-backwards and forwards

Michael Horowitz, Lu Cai, Md Shahidul Islam

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Abstract

This editorial is stimulated by the article by Alqifari *et al* published in the *World Journal of Diabetes* (2024). Alqifari *et al* focus on practical advice for the clinical use of glucagon-like-peptide-1 (GLP-1) receptor agonists (GLP-1RAs) in the management of type 2 diabetes and this editorial provides complementary information. We initially give a brief historical perspective of the development of GLP-1RAs stimulated by recognition of the 'incretin effect', the substantially greater insulin increase to enteral when compared to euglycaemic intravenous glucose, and the identification of the incretin hormones, GIP and GLP-1. In addition to stimulating insulin, GLP-1 reduces postprandial glucose levels by slowing gastric emptying. GLP-1RAs were developed because native GLP-1 has a very short plasma half-life. The majority of current GLP-1RAs are administered by subcutaneous injection once a week. They are potent in glucose lowering without leading to hypoglycaemia, stimulate weight loss in obese individuals and lead to cardiovascular and renal protection. The landscape in relation to GLP-1RAs is broadening rapidly, with different formulations and their combination with other peptides to facilitate both glucose lowering and weight loss. There is a need for more information relating to the effects of GLP-1RAs to induce gastrointestinal symptoms and slow gastric emptying which is likely to allow their use to become more effective and personalised.

Key Words: Glucagon-like-peptide-1; Glucose-dependent insulinotropic peptide; Gastric emptying; Type 2 diabetes

Core Tip: In people who are prescribed a glucagon-like-peptide-1 receptor agonist (GLP-1RA) for management of type 2 diabetes or obesity you should always ask about gastrointestinal symptoms both before and after initiating therapy. Gastrointestinal adverse effects of GLP-1RAs are common, but may not be volunteered.

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INTRODUCTION

The development of glucagon-like-peptide-1 (GLP-1) receptor agonists (GLP-1RAs), which has revolutionised the management of both type 2 diabetes and obesity, represents a story of long-term discovery, driven as much by serendipity as targeted research, with effective, but arguably overdue, translation-the breadth of which has been unanticipated.

BACKGROUND

The so-called 'incretin effect'-that oral, or enteral administration of glucose leads to a much greater (50%-70%) insulin response than isoglycaemic intravenous glucose was reported in 1964 *i.e.* some 60 years ago, but already suggested by the Belgian physiologist La Barre in 1932[1]. Key milestones subsequent to 1964 have been the characterisation of the two 'incretin' hormones-glucose-dependent insulinotropic peptide-GIP (initially termed gastric inhibitory polypeptide) (about 1973) and GLP-1 (about 1985), the demonstration that the 'incretin effect' is attenuated in type 2 diabetes (about 1986) reflecting a markedly diminished insulinotropic effect of GIP (about 1993), which is probably the dominant incretin in health[1], and the landmark observation by Nauck *et al*[2,3] that intravenous administration of GLP-1, in pharmacological concentrations, had the capacity to normalise even markedly elevated blood glucose levels, in type 2 diabetes[2,3]. This latter observation was contrary to expectation based on the outcome of prior animal studies and, importantly, glucose lowering induced by GLP-1 was not associated with induction of hypoglycaemia[2]. The latter reflects the glucose-dependency of the insulinotropic and glucagonostatic actions of GLP-1 and accounts for the safety of GLP-1RAs in relation to their low, to non-existent, potential to induce hypoglycaemia in humans[3].

It was subsequently demonstrated (about 1997) that GLP-1 also slowed the rate of gastric emptying and this, rather than the stimulation of insulin and/or the suppression of glucagon, represented the major mechanism underlying its effect to lower postprandial glucose markedly. Accordingly, GLP-1 was shown to be an enterogastrone, as well as an 'incretin'[4]. Altogether these observations provided a persuasive basis to support the development of drugs based on the actions of GLP-1, rather than GIP, as a glucose lowering therapy. However, it was also appreciated in about 1995, that both GLP-1 and GIP undergo rapid proteolytic degradation in plasma, by a ubiquitous enzyme, dipeptidyl peptide-4 (DPP-4), and that native GLP-1 was, accordingly, unlikely to be used therapeutically because of its short (approximately 2 min) plasma half-life[2]. To overcome the deficiency of native GLP-1 two strategies were developed and have been translated successfully to treat type 2 diabetes-DPP-4 inhibitors, designed prospectively, introduced in 2004 and now widely used, safe oral medications that they have only modest glucose-lowering capacity and are weight neutral and GLP-1RAs, resistant to degradation by DPP-4, administered for the main part subcutaneously and, as will be discussed, with potent effects to reduce elevated blood glucose levels as well as reduce body weight in obese individuals[2].

The first GLP-1RA, exenatide, introduced in 2005 for the management of type 2 diabetes, was, astonishingly, based on exendin-4 isolated from the venom of the Gila monster, *Heloderma suspectum*, a slow-moving lizard native to Southwestern United States. Exenatide exhibits approximately 50% homology to native GLP-1 and is administered subcutaneously twice a day. This has been followed rapidly by the ongoing development of 'designer' molecules (*e.g.* liraglutide, dulaglutide and semaglutide) with increasingly greater efficacy to improve glycaemic control, as well as reduce body weight. There are substantial differences between individual GLP-1RAs, apart from their capacity to reduce blood glucose and body weight, particularly in relation to their duration of action, where they may be classified as either 'short'- or 'longer'-acting'[3].

LIMITATIONS OF PREVIOUS PHARMACOTHERAPY FOR TYPE 2 DIABETES

The significance of the advent of GLP-1RAs should be considered in relation to the substantial limitations in existing approaches to the management of type 2 diabetes. It was appreciated that measurement of glycated haemoglobin (Hb1Ac) was predictive of both the development and progression of the microvascular complications of diabetes, so that management should, ideally, be targeted to achieve a Hb1Ac ≤ 7.0% or even less. Moreover, as a result of the seminal work by Monnier *et al*[5], it was recognised that the contribution of postprandial blood glucose excursions to Hb1Ac is

substantial, and when Hb1Ac is ≤ 7.5% it is the dominant determinant. Approaches to management were also essentially 'glucocentric' with the aim of delaying, if not preventing, microvascular complications. The individual response to glucose-lowering therapy was variable and, at least in most cases, unpredictable and the approach to management was essentially empirical. The use of insulin (and to a lesser extent, sulphonylureas) was, of course, also associated with hypoglycaemia (deleterious and sometimes lethal), increased glucose variability (a potential factor in the risk of micro- and macrovascular complications) and weight gain (in individuals who are characteristically already obese). The therapeutic approaches also had limited, if any, impact on either cardiovascular or renal dysfunction, which were well recognised as major sources of morbidity and mortality. Inherent 'advantages' of GLP-1RAs were, accordingly, that they targeted the 'islet cell defects' in type 2 diabetes of excessive glucagon, and a relative reduction in insulin, secretion, their capacity to normalise both fasting and postprandial hyperglycaemia and the non-existent potential for hypoglycaemia. A further, and major, paradigm shift was the demonstration, in 2016, that liraglutide prevented cardiovascular events and was also renoprotective[6]-the majority of subsequently developed GLP-1RAs have shown similar effects[7]. It should be appreciated that these cardiovascular outcome trials, initiated in about 2008, were mandated by regulatory bodies to test the safety and efficacy of new glucose lowering drugs, and the positive outcomes were generally unanticipated. Several direct and indirect effects may account for the cardio-/reno-protective actions of GLP-1RAs, which appear unrelated to their glucose lowering effect[7].

CONCLUSION

Further developments in GLP-1RAs

The landscape in relation to GLP-1RAs is expanding rapidly. Drugs that are agonists of two or more peptides that are involved in the regulation of glycaemia and/or body weight (*i.e.* a GLP-1RA and at least another compound), such as tirzepatide, a combined GLP-1/GIP agonist, that has recently become available and retatrutide (a combined GLP-1, GIP and glucagon agonist) that is in late phase development[3,8]. Recently, and, contrary to expectation, small molecules that interact with the GLP-1 receptor and are not degraded rapidly when given orally (*e.g.* orforglipron), have been developed and appear effective in both glucose lowering and inducing weight loss[9]. While an oral formulation of semaglutide is available, it has very low bioavailability, even with concomitant use of an absorption enhancer.

The use of GLP-1RAs is being also explored in other diverse disorders, including fatty liver disease and Parkinson's disease[10]. GLP-1RAs may prove useful in the management of postprandial hypotension, a substantial fall (> 20 mm Hg) in systolic blood pressure after a meal, which occurs frequently (approximately 20%) in type 2 diabetes (more commonly than orthostatic hypotension which is well recognised) and predisposes to falls[11]. Postprandial hypotension currently lacks an effective treatment.

Issues relating to the use of GLP-1RAs in type 2 diabetes that should be addressed

The magnitude of the response to GLP-1RAs in type 2 diabetes in terms of both glucose lowering and weight loss is highly variable and largely unexplained-this is not surprising given the empirical design of the majority of clinical trials. This issue represents a focus of the timely review by Alqifari *et al*[12] who provide practical, and useful but, in many cases, unavoidably not evidenced-based, recommendations. Clinicians would benefit greatly by insights as to which patient should be given a GLP-1RA and which GLP-1RA.

Upper gastrointestinal symptoms are the most common adverse event of GLP-1RA therapy (particularly nausea and diarrhoea) and not infrequently (perhaps about 10%) lead to non-adherence and/or treatment discontinuation[3]. Gastrointestinal symptoms, however, also occur frequently in people with type 2 diabetes and the obese who do not have type 2 diabetes[13]. It is regrettable that in nearly all studies relating to GLP-1RAs gastrointestinal symptoms have been assessed solely using participant 'self-report', which is known to be unreliable, rather than simple, validated measures that are readily available and used extensively in the assessment of functional gastrointestinal disorders (*e.g.* irritable bowel syndrome and functional dyspepsia[14]. The relevance of symptom induction to weight loss induced by GLP-1RAs, accordingly, still remains uncertain. The impact of GLP-1RAs to slow gastric emptying, which is integral to their capacity to reduce postprandial glycaemic excursions, also requires clarification. 'Short-acting' GLP-1RAs (*i.e.* exenatide BID and lixisenatide) have been shown, using accurate methods, to slow gastric emptying markedly but variably. This slowing occurs in doses substantially less than used in the management of type 2 diabetes[13,15] and is predictive of the reduction in postprandial glucose[13]. It was assumed (without accurate measurement) that 'longer-acting' GLP-1RAs did not have sustained effect to slow gastric emptying, but this concept has recently been shown to be incorrect-liraglutide[16], exenatide QW[17] and semaglutide sc[18] all slow gastric emptying substantially and, like 'short-acting' GLP-1RAs, variably, with longer-term administration. Gastric emptying is also frequently delayed in longstanding, complicated type 2 diabetes per se, but cannot be predicted on the basis of symptoms[13]. This issue has assumed even greater importance with recent reports of retained gastric content, despite adherence to recommended periods of fasting in individuals using long-acting GLP-1RAs with cases of aspiration[19]. This has stimulated recent guidelines for the use of GLP-1RAs prior to surgery/endoscopic procedures, which unavoidably lack a strong evidence base. Assessment of their effect on gastric emptying, using a precise technique, should be part of the routine development of GLP-1RAs[14].

FOOTNOTES

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REVIEW

Practical guide: Glucagon-like peptide-1 and dual glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 receptor agonists in diabetes mellitus

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Abstract

In 2005, exenatide became the first approved glucagon-like peptide-1 receptor agonist (GLP-1 RA) for type 2 diabetes mellitus (T2DM). Since then, numerous GLP-1 RAs have been approved, including tirzepatide, a novel dual glucose-dependent insulinotropic polypeptide (GIP)/GLP-1 RA, which was approved in 2022. This class of drugs is considered safe with no hypoglycemia risk, making it a

common second-line choice after metformin for treating T2DM. Various considerations can make selecting and switching between different GLP-1 RAs challenging. Our study aims to provide a comprehensive guide for the usage of GLP-1 RAs and dual GIP and GLP-1 RAs for the management of T2DM.

Key Words: Glucagon-like peptide-1 receptor agonist; Diabetes mellitus; Metabolic syndrome; Dual glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 receptor agonist; Clinical practice; Endocrinology

Core Tip: Various glucagon-like peptide-1 receptor agonists (GLP-1 RAs) are available for the management of type 2 diabetes mellitus including short-acting and long-acting injectables as well as one agent as an oral tablet. Furthermore, dual glucose-dependent insulinotropic polypeptide (GIP) and GLP-1 RAs have now emerged as the newest addition to the long-acting injectables. With the availability of various options, the complexity of choosing, titrating, and switching between agents, especially in certain patient populations, has become increasingly challenging. We aim to provide a comprehensive practical clinical guide for practitioners regarding GLP-1 RA and dual GIP and GLP-1 RA use in everyday clinical practice.

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INTRODUCTION

The glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are hormones that are secreted in the intestine within minutes in response to food intake and are collectively known as incretin hormones[1]. They lower glucose levels and maintain a state of glucose homeostasis by enhancing insulin secretion following meals as well as by increasing the cells' sensitivity to insulin[2,3]. Additionally, these agents delay gastric emptying, a factor that significantly influences the pace of alcohol absorption, and a determinant of plasma ethanol response. When gastric emptying is slower, absorption is delayed, leading to lower peak blood alcohol concentration[4,5].

Therefore, research focused on developing drugs that simulated the action of these hormones. In 2005, exenatide became the first approved GLP-1 receptor agonist (GLP-1 RA) for the treatment of type 2 diabetes mellitus (T2DM)[6]. Later, more GLP-1 RAs were approved, and they showed good results in improving glycemic control and reducing weight[7,8]. Given its proven efficacy in weight reduction, liraglutide was approved in 2021 as a treatment for obesity, making it the first GLP-1 RA approved in that domain[9]. Moreover, tirzepatide is a novel drug with the dual effect of both GIP and GLP-1 RA actions which has been recently approved for treating T2DM[10]. It is available as a weekly subcutaneous injection and has shown positive results in controlling glucose levels and lowering the glycated hemoglobin level (HbA1C) as compared to other medications[11-13]. Currently, it is important to highlight that long-acting GLP-1 RA have been predominantly replaced short-acting GLP-1 RA, despite the fact that exenatide BID is now considered off-patent.

According to the recent recommendations by the American Diabetes Association/European Association for the Study of Diabetes and the American Association of Clinical Endocrinology, there is an agreement to consider GLP-1 RAs as a second-line therapy for patients with T2DM who did not show improvement with metformin[14-17]. The addition of GLP-1 RAs is also recommended for prediabetic patients for whom a normoglycemic state has not been achieved with lifestyle changes and/or metformin monotherapy[17]. Likewise, for patients with an initial HbA1C level < 7.5% as well, GLP-1 RAs are recommended as a second-line agent[17]. On the other hand, for patients with an entry HbA1C level of 7.5%, a strategy of dual therapy including GLP-1 RAs as a first-line therapy in addition to metformin is recommended [16]. GLP-1 RAs, along with sodium-glucose cotransporter 2 inhibitors, are considered the first-line add-on drugs in diabetic patients who have cardiovascular risk or chronic kidney disease[14-16]. Furthermore, GLP-1 RAs are indicated as a first-line intervention when metformin is contraindicated[14-16]. GLP-1 RAs are recommended due to their ability to enhance weight loss, lower the risk of hypoglycemia, provide cardiovascular and kidney-protective benefits, and reduce the incidence of microvascular complications of T2DM[14-19].

Several GLP-1 RAs are available, each with varying characteristics, such as route of administration, frequency, dosing, cost, and dosage. Several factors may necessitate a healthcare professional to switch between different GLP-1 RAs. Recent literature indicates that there is a need for more information regarding specific GLP-1 RAs and dual GIP/GLP-1 RAs (*e.g.*, tirzepatide). Therefore, this paper aims to fill these gaps by providing comprehensive guidance for the utilization of GLP-1 RAs and dual GIP/GLP-1 RAs. Specifically, we aim to develop clear practical guidance that will enable healthcare professionals to know how and when to utilize and switch between GLP-1 RAs and dual GIP/GLP-1 RAs.

LITERATURE REVIEW

We searched PubMed using the terms GLP-1 AND (switch OR switching OR switched); and GLP-1 AND (once-daily OR "once daily") AND (once-weekly OR "once weekly") AND GIP AND dual GIP and GLP-1 with no lower limit set for the date, using MeSH and free text terms to match relevant articles. We included all types of articles with publication dates starting from September 2003 to September 2023. We restricted the search to human studies and only those that were in the English language. These searches yielded 58, 78, and 25 results, respectively. Abstracts of the literature thus retrieved were then manually reviewed by two experts to identify the relevant articles on utilization and switching between different GLP-1 RAs and dual GIP/GLP-1 RAs.

OVERVIEW OF GLP-1 AND DUAL GIP AND GLP-1 RA

Characteristics and clinical implications

GLP-1 RAs and dual GIP/GLP-1 RAs available in the market exhibit many similarities and variations. Despite being in the same class, GLP-1 RAs vary according to their pharmacological characteristics, effectiveness, and safety profiles[20-27]. GLP-1 RAs and dual GIP/GLP-1 RAs commonly available are listed in Table 1.

GLP-1 RAs showed efficacy in T2DM and obesity management[20-27]. The native intrinsic forms of human GLP-1 RAs have a very short half-life as dipeptidyl peptidase-4 degrades them rapidly after just a few minutes of being released into the bloodstream[28]. Consequently, structural modifications were made by removing amino acids or adding fatty acid chains to confer resistance to enzymatic degradation[28].

A newly synthesized analog, tirzepatide, which has a dual agonism on GLP-1 and GIP receptors, has been developed. It has a unique structure as a linear peptide with a fatty di-acid chain attached to it[29]. This novel compound has been found to significantly improve glycemic control and manage inadequate response in patients receiving insulin glargine [30].

Most of the newly developed GLP-1 RAs can be administered subcutaneously *via* injections, except for the short form of semaglutide that is given orally. Exenatide is a short-acting agent taken in two daily doses, while oral semaglutide, lixisenatide, and liraglutide are all given once daily. Based on their extended half-life, the remaining medications are prescribed once-weekly[21-27].

Characteristics of semaglutide: Semaglutide, a once-weekly injectable medication categorized as a specific GLP-1 RA, has gained approval for managing T2DM at dosages of up to 1 mg. Clinical studies conducted on individuals receiving semaglutide revealed significant average decreases in HbA1C of up to 1.8% and substantial average reductions in body weight of up to 6.5 kg[31].

Characteristics of tirzepatide: Tirzepatide, a unique dual-action agent, functions both as a GIP and a GLP-1 RA and is a medication newly approved by the United States Food and Drug Administration (FDA) for managing T2DM. Its chemical structure is predominantly derived from the amino acid sequence of GIPs and incorporates a C20 fatty di-acid component [32]. Tirzepatide has an approximate bioavailability of 80%, and the time that it takes to reach its highest concentration in the bloodstream can vary, spanning from 8 to 72 h, while its average apparent steady-state volume of distribution is roughly 10.3 L. It is important to note that tirzepatide exhibits high binding to plasma albumin, with approximately 99% of the drug being plasma protein bound in the bloodstream. Upon injection, the peptide structure undergoes a proteolytic cleavage, marking the degradation and metabolism process of the drug. The C20 fatty di-acid component also experiences beta-oxidation and amide hydrolysis[32,33]. Tirzepatide has a half-life of 5 d, which enables dosing on a once-weekly basis. It is eliminated from the body as metabolites through urine and feces. Tirzepatide is administered through subcutaneous injection and is not currently available in oral form. It is available in several dosage options: 2.5 mg/0.5 mL, 5 mg/0.5 mL, 7.5 mg/0.5 mL, 10 mg/0.5 mL, 12.5 mg/0.5 mL, and 15 mg/0.5 mL. The starting dose is 2.5 mg for treatment initiation not intended for glycemic control and titrated to 5 mg after 4 wk[32].

In a 40-wk clinical trial involving 917 individuals diagnosed with T2DM comparing tirzepatide to insulin glargine, it was observed that tirzepatide led to a greater average reduction in HbA1C levels compared to insulin glargine. Furthermore, a smaller percentage of patients experienced hypoglycemia, defined as glucose levels below 54 mg/dL, when using tirzepatide as opposed to insulin glargine[33]. Moreover, there was a mean reduction in body weight of 5 kg, 7 kg, and 7.2 kg for individuals taking 5 mg, 10 mg, and 15 mg of tirzepatide, respectively[33]. Tirzepatide does not appear to elevate the risk of major cardiovascular events. For instance, a meta-analysis of seven phase II and III trials comparing tirzepatide to either a placebo or an active comparator showed no increase in the composite cardiovascular endpoints associated with tirzepatide[33].

Regarding the tirzepatide age threshold, its distribution in the SURPASS 1-5 studies varied due to distinct inclusion and exclusion criteria with no upper age limit specified for participants. In the combined dataset from seven clinical trials, 30.1% of the patients who received tirzepatide were aged 65 years or older, and 4.1% were 75 years or older at the beginning of the study. Overall, there were no significant differences in terms of safety or effectiveness observed between the older patients and their younger counterparts. However, it is important to note the possibility that some older individuals who may exhibit heightened sensitivity to the treatment cannot be definitively ruled out[27]. Additionally, as the SURPASS 1-5 trials excluded individuals under 18 years of age, a separate trial (NCT05260021) is set to assess the effects of tirzepatide in pediatric and adolescent participants aged 10 to 18 years who have type 2 diabetes[34].

Table 1 Characteristics of glucagon-like peptide-1 receptor agonists

Name	MOA	ROA	Available doses	Frequency	HbA1C reduction	Elimination and dose adjustment	Half-life	Dosing instructions	Drug-interactions
Lixisenatide	GLP-1 receptor agonist	SC	Initial: 14 doses of 10 μg per dose Followed by: 14 doses of 20 μg per dose	Once daily	-0.65 (after 12 wk of monotherapy) compared with placebo, -0.46 (in 24 wk), -0.27 in combination with metformin +/- sulfonylurea (in 24 wk), -0.48 in combination with pioglitazone +/- metformin (in 24 wk), -0.28 in combination with insulin glargine and metformin +/- thiazolidinediones (in 24 wk)	Renal elimination, dependent on GFR Insufficient data on ESRD. No dose adjustment for mild or moderate renal impairment	Approximately 3 h	1 h before meals Oral medications 1 h before injection	Delayed gastric emptying, decreased absorption and decreased effectiveness of some oral medications
Exenatide	GLP-1 receptor agonist	SC	Initial: 60 doses of 5 μg per dose Followed by: 60 doses of 10 μg per dose	BID	After 30 wk: -0.5 for 5 μg once daily, -0.7 for 10 μg once daily, -0.5 for 5 μg BID, -0.9 for 5 μg BID -0.8 and -1.0 for 5 and 10 μg, respectively, in combination with metformin and sulfonylurea	Renal elimination Avoided in ESRD and severe renal impairment. No dose adjustment for mild renal impairment	2.4 h	1 h before the two main meals The meals must be 6 h apart	Increased INR in patients with warfarin
Exenatide extended release	GLP-1 receptor agonist	SC	2 mg	Every 7 d	No significant difference from metformin and pioglitazone after 26 wk, -0.39 as compared to sitagliptin use -0.63 in combination with metformin as compared to sitagliptin, and -0.32 when compared to pioglitazone (in 26 wk), -0.64 in combination with glargine (in 28 wk)	Renal elimination Avoided in ESRD and severe renal impairment	2-4 wk	At any time of the day	Increased INR in patients with warfarin May impact the absorption of oral medications
Liraglutide	GLP-1 receptor agonist	SC	Initial: 0.6 mg for 1 wk Followed by: Increase to 1.2 mg If additional glycemic control needed increase to 1.8 mg after 1 wk	Once daily	-0.3 and -0.6 for 1.2 and 1.8 mg, respectively, after 52 wk compared to glimepiride. Both doses showed -1.1 when combined with metformin compared to placebo in 26-wk trial. -0.3 and -0.6 for 1.2 and 1.8 mg, respectively, in 26-wk trial when combined with metformin compared with sitagliptin; -1.06 in combination with metformin and basal insulin compared to placebo	No specific organ as main part of elimination No dose adjustment is needed for renal disease Should be used cautiously in patients with hepatic impairment as sufficient data is absent for this population	13 h	At any time of the day	Delayed gastric emptying
Dulaglutide	GLP-1 receptor agonist	SC	0.75 mg 1.5 mg if additional	Every 7 d	-0.5 and -0.7 for 0.75 and 1.5 mg, respectively, when compared to	No specific organ as main part of elimination	5 d	At any time of the day	Potential decrease in absorption of oral

			glycemic control is needed Increase the dose by 1.5 mg, at least 4 wk after the previous dose, maximum dose 4.5 mg		sitagliptin in 52-wk trial; -1.1 for 1.5 mg combined with glimepiride when compared to placebo; -0.7 for 1.5 mg combined with basal insulin in 26-wk trial				medications
Tirzepatide	Glucose-dependent insulinotropic polypeptide and GLP-1 receptor agonist	SC	Initial: 2.5 mg	Every 7 d	-1.7, -1.6, and -1.6 for 5, 10, and 15 mg, respectively, when compared to placebo in 40-wk trial; -0.2, -0.4, and -0.5 for 5, 10, and 15 mg, respectively, when compared to semaglutide in 40-wk trial; -0.6, -0.8, and -0.9 for 5, 10, and 15 mg, respectively, when compared to insulin degludec in 52 wk	Hepatic and renal elimination	5 d	At any time of the day	Potential decrease in absorption of oral medications
			After 4 wk increase the dose to 5 mg		-0.7, -0.9, and -1 for 5, 10, and 15 mg, respectively, when compared to insulin glargine in 52 wk	No dose adjustment is needed for renal and hepatic diseases			
			Increase the dose at 2.5 mg, at least 4 wk apart from the previous dose, maximum dose 15 mg						
Semaglutide	GLP-1 receptor agonist	SC	Initial dose 0.25 mg After 4 wk increase the dose to 0.5 mg If additional glycemic control needed increase to 1 mg after 4 wk, and if further control is required increase to 2 mg after 4 wk of 1 mg dose	Every 7 d	-1.4 and -1.6 for 0.5, and 1 mg, respectively, when compared to placebo in 30 wk trial; -0.6 and -0.8 for 0.5 and 1 mg, respectively, when compared to placebo in 56-wk trial; -0.5 for 1 mg in comparison with exenatide in combination with metformin or metformin with sulfonylurea	Hepatic and renal elimination No dose adjustment is needed for renal and hepatic diseases	1 wk	At any time of the day	Potential decrease in absorption of oral medications
Oral Semaglutide	GLP-1 receptor agonist	Oral	Initial dose: 3 mg for 30 d Followed by: 7 mg	Once daily	-0.9 and -1.1 for 7 and 14 mg, respectively, when compared to placebo in 26 wk trial	Hepatic and renal elimination No dose adjustment needed for renal and hepatic diseases	1 wk	30 min before any oral intake	Potential decrease in absorption of oral medications

	If additional glycemic control needed increased to 14 mg after 30 d of 7 mg dose	-0.3 and -0.5 for 7 and 14 mg, respectively, when compared to sitagliptin in 26-wk trial; -0.1 for 14 mg dose when compared with liraglutide; 0.9 and -1.2 for 7 and 14 mg, respectively, combined with insulin when compared to placebo in 26-wk trial

MOA: Mechanism of action; ROA: Route of administration; HbA1C: Glycated hemoglobin; GFR: Glomerulus filtration rate; ESRD: End-stage renal disease; BID: Twice daily; GLP-1: Glucagon-like peptide-1; INR: International normalized ration; SC: Subcutaneous injection.

Comparison of tirzepatide with semaglutide: In a recent trial, tirzepatide, administered at a dose of 5 mg, 10 mg, or 15 mg, exhibited noninferiority and superiority in comparison to injectable semaglutide at a dose of 1 mg. Tirzepatide reduced the HbA1C in patients diagnosed with T2DM who were also taking metformin as part of their treatment regimen [32]. The reductions in body weight were more significant in patients treated with tirzepatide when compared to those receiving injectable semaglutide. There was a difference of -1.9 kg, -3.6 kg, and -5.5 kg for tirzepatide at doses of 5 mg, 10 mg, and 15 mg, respectively, compared to injectable semaglutide[32]. Dual GIP/GLP-1 RA therapy seems to lead to more significant weight loss compared to GLP-1 RA alone. In a 40-wk clinical trial that compared tirzepatide with semaglutide, both administered once-weekly *via* subcutaneous injection, it was observed that tirzepatide resulted in a greater average reduction in body weight when compared to semaglutide[32,35].

In the same study, among patients with T2DM, it was found that tirzepatide achieved a superior reduction in HbA1C levels compared to semaglutide[32]. In patients who were administered tirzepatide, the risk of hypoglycemia (defined as a blood glucose level below 54 mg/dL) was reported as 0.6% in the 5-mg group, 0.2% in the 10-mg group, and 1.7% in the 15-mg group. In contrast, the risk of hypoglycemia was observed in 0.4% of individuals who received 1 mg of injectable semaglutide[32]. The most frequent adverse events reported were related to the gastrointestinal (GI) system and were generally of mild to moderate severity in both the tirzepatide and injectable semaglutide groups. Specifically, nausea was reported in 17% to 22% of patients treated with tirzepatide and in 18% of those receiving semaglutide. Diarrhea was reported by 13% to 16% of tirzepatide-treated patients and 12% of those taking semaglutide. Vomiting was experienced by 6% to 10% of tirzepatide recipients and 8% of semaglutide recipients, while a reduced appetite was noted in 7% to 9% of tirzepatide-treated patients and 5% of those on 1 mg semaglutide. In another trial comparing tirzepatide with semaglutide, the incidence of adverse GI effects was similar between the two groups[32,35]. Serious adverse events were documented in 5% to 7% of patients receiving tirzepatide and in 3% of those taking injectable semaglutide. Hypersensitivity reactions were observed in 1.7% to 2.8% of patients treated with tirzepatide and in 2.3% of those treated with semaglutide. Injection-site reactions were reported in 1.9% to 4.5% of patients receiving tirzepatide and 0.2% of those receiving semaglutide. Notably, these injection-site and hypersensitivity reactions were generally of mild to moderate severity, and no severe cases of either were reported[32,35].

Interactions of dual GIP and GLP-1 RAs with other medications

Drug interactions can significantly impact the effectiveness and safety of drug therapy. The therapeutic efficacy of tirzepatide can be increased when used in combination with insulin secretagogues such as sulfonylureas or insulin and oral antidiabetic agents. Nevertheless, this combination also leads to a higher risk of hypoglycemia.

GLP-1 RAs slow down gastric emptying, which may induce pharmacokinetic changes in interacting drugs such as acetaminophen, digoxin, warfarin, oral contraceptives, metformin, statins, angiotensin-converting enzyme inhibitors, and griseofulvin. Despite these interactions, they are generally deemed clinically insignificant, and dosage adjustments are unnecessary when using most of these drugs concurrently with GLP-1 Ras[35,36]. However, it is important to note that the simultaneous administration of warfarin with GLP-1 RAs may result in an increased international normalized ratio (INR), and although this effect is not significant, close monitoring of the INR is advised considering warfarin's narrow therapeutic index[35,36]. Furthermore, to avoid any delay in drug absorption, it is recommended to take interacting drugs approximately 1 h before administering GLP-1 Ras[35].

Cardioprotective effect of dual GIP and GLP-1 RAs

GLP-1 RAs provide cardioprotective effects through several mechanisms. These agents lower systolic blood pressure by around 2-3 mmHg, reduce endothelial inflammation and oxidative stress, and promote the induction of endothelial nitric oxide synthase, which increases nitric oxide availability[37,38]. Additionally, GLP-1 RAs promote natriuresis and diuresis by inhibiting the sodium-hydrogen exchanger 3 of the renal proximal tubular cells, which could partly account for the blood pressure-lowering effects[39].

GLP-1 RAs display anti-inflammatory properties by reducing the production of proinflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-6, and IL-1β and the C-reactive protein levels[40,41]. Furthermore, GLP-1 RAs decrease the expression of adhesion molecules (specifically vascular cellular adhesion molecule-1, intercellular adhesion molecule-1, and P-selectin) on the endothelial cell surfaces, consequently reducing adhesion and migration of inflam-

matory cells, particularly monocytes and neutrophils, through the vascular wall, which reduces the formation of atherosclerotic plaque[42]. Moreover, GLP-1 RAs demonstrated anti-aggregation effects on the activity of murine and human platelets in numerous preclinical studies[43].

Cardiovascular outcome trials of dual GIP and GLP-1 RAs

Due to the strong association between T2DM and cardiovascular complications, clinical studies must establish the cardiovascular safety of any drug for T2DM to obtain United States FDA approval. This has led to many cardiovascular outcome trials involving innovative glucose-lowering medications like GLP-1 RAs.

The Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial assessed the cardiovascular safety of liraglutide in 9340 patients with T2DM and high cardiovascular risk. Participants were randomly assigned to receive either 1.8 mg of liraglutide or a placebo once daily and observed for 3.5 years. Results showed a 13% reduction in major adverse cardiovascular events (MACEs), a 15% lower overall mortality, and a 22% reduction in cardiovascular-related deaths among those receiving liraglutide treatment compared to the placebo group. However, no significant differences were noted between the groups in nonfatal myocardial infarctions or nonfatal strokes [44].

Semaglutide has been the focus of two extensive cardiovascular outcome trials: The Semaglutide Unabated Sustainability in Treatment of Type 2 Diabetes 6 (SUSTAIN-6) trial and the Oral Semaglutide and Cardiovascular Outcomes in Patients with T2DM (PIONEER 6) trial. In the SUSTAIN-6 trial, 3297 individuals with T2DM and elevated cardiovascular risk, 83% with established cardiovascular disease, were randomly assigned to receive subcutaneous injections of once-weekly semaglutide at a dose of 0.5 mg or 1 mg, or a placebo. Over a median period of 2.1 years, the trial revealed a significant 26% reduction in MACEs in semaglutide-treated subjects, primarily driven by a substantial decrease in nonfatal stroke events. It is noteworthy to mention that semaglutide-treated individuals reported a higher incidence of complications associated with retinopathy[45].

On the other hand, in the PIONEER 6 trial, which assessed oral semaglutide, the administration of a once-daily 14 mg dose did not result in a reduced rate of MACEs, nonfatal myocardial infarctions, or nonfatal strokes. However, a significant reduction in cardiovascular deaths was evident among participants who received oral semaglutide[31].

In the Effect of Efpeglenatide on Cardiovascular Outcomes (AMPLITUDE-O) trial, which included 4076 patients with T2DM and either prior cardiovascular disease or existing kidney disease along with at least one additional cardiovascular risk factor, the occurrence of MACEs was significantly reduced by 27% in those who received efpeglenatide compared to a placebo. Furthermore, the efpeglenatide group exhibited a notably reduced risk of hospitalization for heart failure[46].

The HARMONY Outcomes trial involved 9463 individuals with T2DM and established cardiovascular disease who were randomly assigned to receive either a 30 mg weekly dose of albiglutide or a placebo. After a median follow-up period of 1.5 years, the albiglutide group exhibited a 22% reduced risk of MACEs. However, there was no statistically significant difference in the risk of cardiovascular, all-cause mortality, and stroke[47].

In the SURPASS-4 trial, 2002 participants were randomly assigned to receive either tirzepatide at varying strengths (5 mg, 10 mg, or 15 mg) or insulin glargine. The study observed participants experiencing adjudicated MACEs, including cardiovascular death, myocardial infarction, stroke, and hospitalization for unstable angina. Importantly, the occurrence of these events was not higher in the tirzepatide group when compared to the glargine group and it was concluded that tirzepatide treatment was not associated with increased cardiovascular risk[48].

Additionally, the ongoing SUMMIT trial aims to evaluate tirzepatide's effects on individuals with both obesity and heart failure with preserved ejection fraction. Participants will receive tirzepatide or a placebo for 52 wk, with the primary outcome being a composite endpoint that includes mortality, heart failure events, exercise capacity, and heart failure symptoms[49]. In summary, the outcomes of the previously mentioned trials provided strong support for the utilization of dual GIP and GLP-1 RAs in individuals with T2DM and established or significant risk of cardiovascular disease. Cardiovascular outcome trials are listed in Table 2.

Nephroprotective effect of dual GIP and GLP-1 RAs

GLP-1 RAs exhibit nephroprotective effects independently of their impact on blood glucose levels. In addition to inducing natriuresis and diuresis, GLP-1 RAs demonstrate antioxidative and anti-inflammatory properties. One of these involves the activation of the cyclic adenosine monophosphate-protein kinase A pathway, reducing the nicotinamide adenine dinucleotide phosphate oxidative activity and the reactive oxygen species production in the diabetic kidney[18].

Furthermore, GLP-1 RAs promote the reduction of mesangial expansion and the elevation of nitric oxide levels within the glomeruli, ultimately improving glomerular filtration and hemodynamic function, all of which help inhibit the progression of diabetic kidney disease. Moreover, GLP-1 RAs have been shown to decrease markers of renal renin-angiotensin-aldosterone system (RAAS) activation, including angiotensin II levels, and mitigate its detrimental effects within the glomerulus. However, comprehensive data regarding the effects of acute or long-term GLP-1 RA treatment on circulating RAAS components are still lacking. Natriuresis, lowering plasma renin activity and renal oxidative stress, improving blood pressure, and glycemic control collectively contribute to the anti-albuminuric effects observed with GLP-1 Ras[18].

In the LEADER trial, liraglutide reduced the incidence of new or worsening nephropathy by 22% and showed a slight deceleration in the decline of the estimated glomerular filtration rate (eGFR) over time when compared to a placebo. In the SUSTAIN-6 trial, semaglutide reduced the risk of persistent macroalbuminuria. However, both trials revealed no significant differences in more severe renal outcomes, such as doubling of serum creatinine levels or the need for renal replacement therapy[44,45]. In the SURPASS-4 trial, tirzepatide significantly slowed the rate of eGFR decline, reduced the urinary albumin-to-creatinine ratio, and reduced the incidence of the composite kidney endpoint (time to first occurrence of eGFR decline of at least 40% from baseline, ESRD, kidney failure related death, or new-onset macroalbuminuria) in

Table 2 Cardiovascular outcome trials of glucagon-like peptide-1 and dual glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 receptor agonists

Trial name	No. of patients	Study population	Active comparator	Follow-up	Outcomes
LEADER	9340	T2DM, ≥ 50 yr with established CVD, or age ≥ 60 yr with CV risk factors	1.8 mg of liraglutide once-daily SC	3.8 yr	13% reduction in MACEs; 15% reduction in overall mortality; 22% reduction in CV-related deaths
SUSTAIN-6	3297	T2DM, ≥ 50 yr with established CVD, or CKD ≥ stage 3, or age ≥ 60 yr with CV risk factors	0.5 mg or 1.0 mg semaglutide once-weekly SC	2.1 yr	26% reduction in MACEs; 39% reduction in non-fatal stroke
PIONEER 6	3183	T2DM, ≥ 50 yr with established CVD, or CKD ≥ stage 3, or age ≥ 60 yr with CV risk factors	14 mg of semaglutide once-daily oral	1.3 yr	No significant reduction in MACEs; 51% significant reduction in CV-related deaths
AMPLITUDE-O	4076	T2DM, ≥ 50 yr with established CVD, or CKD ≥ stage 3 with CV risk factors	4 or 6 mg of efpeglenatide once-weekly SC	1.81 yr	27% reduction in MACEs; reduced risk of hospitalization for heart failure
HARMONY	9463	T2DM, age ≥ 40 yr with CVD	30-50 mg of albiglutide once-weekly SC	1.6 yr	22% reduction in MACEs
SURPASS-4	2002	T2DM, ≥ 18 yr with established CVD, or with CV risk factors	5 mg, 10 mg, or 15 mg of tirzepatide once-weekly SC	2 yr	Tirzepatide treatment was not associated with increased CV risk

MACEs: Major adverse cardiac events, including cardiovascular death, non-fatal myocardial infarction, non-fatal stroke, and hospitalization for unstable angina; CKD: Chronic kidney disease; CV: Cardiovascular; CVD: Cardiovascular disease; T2DM: Type 2 diabetes mellitus; SC: Subcutaneous; LEADER: Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results; SUSTAIN-6: Semaglutide Unabated Sustainability in Treatment of Type 2 Diabetes 6.

patients with T2DM compared to insulin glargine[48].

Contraindications and precautions for GLP-1 and dual GIP and GLP-1 RA use

Pancreatitis risk: Although the exact mechanism remains largely unidentified, cases of acute pancreatitis, including potentially fatal hemorrhagic and necrotizing forms, have been documented among users of GLP-1 RAs. Meanwhile, it is unclear whether a direct cause-and-effect relationship exists between GLP-1 RAs and pancreatitis or pancreatic cancer. Since the data remains unclear, patients with a history of pancreatitis should not be treated with GLP-1 Ras[50].

T1DM: Certain beneficial effects of GLP-1 RAs, such as reducing glucagon levels and promoting weight loss, are not reliant on the functioning of islet cells. This could potentially be advantageous for certain individuals with T1DM. However, as of now, until more data becomes available, studies refrain from prescribing GLP-1 RAs for patients with T1DM[51].

Renal impairment: In patients with severe renal impairment (eGFR 15 to 29 mL/min) and end-stage renal disease, lixisenatide and albiglutide are not recommended as there is limited experience with these drugs in this population[52,53]. However, a study showed that a 5 mg dose of tirzepatide was tolerated in patients with renal impairment, and no effect on the pharmacokinetics was observed[54].

Gastroparesis & inflammatory bowel disease: Patients with gastroparesis and inflammatory bowel disease should avoid GLP-1 analogs. It's crucial to acknowledge the absence of precise measurements for gastric emptying using appropriate methodologies when it comes to longer-acting GLP-1 RAs. Moreover, there should be recognition of the suboptimal assessment of gastrointestinal adverse effects relying on self-reported information[55]. Furthermore, the accurate diagnosis of gastroparesis relies on direct measurement, with Scintigraphy remaining the 'gold-standard' technique[56].

The mechanisms by which GLP-1 and incretin-based therapies affect gut motility are not fully understood but research conducted on the duodenum and colon of rodents suggests that GLP-1 can reduce excitatory cholinergic neurotransmission in the enteric nervous system by acting on presynaptic GLP-1 receptors, which in turn modulate the release of nitric oxide[57]. Therefore, GLP-1 RAs could potentially be employed as a treatment to relieve symptoms in individuals with irritable bowel syndrome *via* decreasing motility in the intra-duodenal-jejunal region and inhibiting the migrating motor complex in both healthy individuals and patients[58].

Thyroid cancer: Concerns exist regarding a potential link between GLP-1 RAs and thyroid cancer, supported by rodent studies showing associations with thyroid C-cell proliferation and neoplasia. Conflicting evidence and controversies have arisen from clinical trials and databases regarding this matter in human studies. In humans, the GLP-1 receptor was identified in 18% of papillary thyroid carcinomas and 33% of control thyroid lobes, including neoplastic and hyperplastic lesions of thyroid C-cells[35,50,59]. Additionally, GLP-1 may function through the phosphoinositol-3 kinase/AKT serine/threonine kinase pathway and/or mitogen-activated protein kinase/extracellular signal-regulated kinase pathway, which

play a crucial role in controlling cell growth and proliferation and are closely associated with cancer, including papillary thyroid carcinoma[60]. Additionally, a recent study identified an elevated risk of all types of thyroid cancers and medullary thyroid cancer associated with the use of GLP-1 RAs, particularly notable after 1-3 years of treatment duration [61]. However, a recent meta-analysis study revealed a significant 28% increase in the overall risk of thyroid disorders when using GLP-1 RAs compared to placebos or other interventions but no significant correlation with thyroid cancer was identified[62]. Although evidence in human studies remains inconclusive, GLP-1 RAs are contraindicated in patients with a personal or family history of medullary thyroid carcinoma or multiple endocrine neoplasia syndrome type 2[35, 62].

Hypersensitivity and other contraindications: While hypersensitivity reactions to GLP-1 RAs are rare, in cases where an individual has a history of such a reaction to any GLP-1 RA, it is typically advisable to opt for an alternative glucose-lowering agent that does not belong to the GLP-1 RA class. Furthermore, other relative contraindications may exist, such as acute gallbladder diseases like acute cholecystitis with GLP-1 RA in general or diabetic retinopathy specifically with semaglutide use[63,64].

Cost burden of dual GIP and GLP-1 RAs

Cost considerations play a crucial role in the selection of medications and switching between them. A cost-effectiveness analysis in Saudi Arabia found that semaglutide was the most financially advantageous option, with the lowest cost of achieving glycemic control to reach target HbA1C levels compared to other GLP-1 RAs (liraglutide, dulaglutide, exenatide, and lixisenatide)[65]. A study in Taiwan found that GLP-1 RA therapy had higher costs per patient compared to insulin from the payer perspective, but that the GLP-1 RA group incurred lower costs than the insulin group in the healthcare sector, primarily due to decreased expenses related to emergency visits and in-patient admissions. Despite increased drug costs, real-world GLP-1 RA usage showed cost-effectiveness, with lower healthcare costs linked to lower mortality and hypoglycemia-related hospitalizations[66]. In a United States database study, once-weekly dulaglutide had similar diabetes-related total costs to daily liraglutide but was associated with higher costs compared to once-weekly exenatide[67]. In another United States study, it was demonstrated that once-weekly semaglutide at doses of 0.5 mg and 1.0 mg outperforms exenatide ER and dulaglutide in terms of cost-effectiveness for achieving both individual and combined treatment endpoints. This includes improvements in glycemic control, reduction in body weight, and avoidance of hypoglycemia. Consequently, the study suggests that once-weekly semaglutide at these specified doses presents a favorable economic proposition in the United States, especially for the achievement of comprehensive treatment objectives in individuals with type 2 diabetes[68].

SWITCHING BETWEEN DIFFERENT GLP-1 AND DUAL GLP-1/GIP RAS

There is a lack of consensus on how to switch between different GLP-1 and dual GLP-1/GIP RA agonists, and no evidence or guidelines to follow for switching, so we rely on clinical practice experiences from members in this research group in different settings both inside and outside Saudi Arabia. However, further study in this regard is warranted. Switching between GLP-1 and dual GLP-1/GIP RAs may be required for several reasons including drug availability, adherence, patient preference, cost, drug tolerability, side effects, and efficacy. When switching from one agent to another, it is crucial to first address the reason for switching, and then, based on the duration and dose of the previous GLP-1 RA or dual GLP-1/GIP RA, along with the patient's experience, especially the GI side effects, an individualized approach is recommended[69].

For those with GI side effects, we consider stepwise medication withdrawal to determine the causative agent and facilitate medication tolerance before switching. We ensure that all recommended measures to mitigate GI side effects have been taken, such as ensuring that the patient is receiving the recommended dosage of the GLP-1 or dual GLP-1/GIP RA as dose reduction can frequently reduce or eliminate GI side effects; ensuring that dietary recommendations are followed (eating smaller portions and avoiding high-fat meals); and trying other mitigating measures such as implementing a short-term liquid diet or using natural antinausea remedies like ginger or peppermint[69].

For patients who cannot tolerate GLP-1 or dual GLP-1/GIP RAs despite the mitigating measures, we recommend waiting until symptoms subside, then initiating the new GLP-1 or dual GLP-1/GIP RA therapy at the lowest dose, and then considering a slower dose up-titration[70]. For patients who can tolerate GLP-1 or dual GLP-1/GIP RAs but are changing their medication for other reasons, starting the new medication at an equivalent dose is a reasonable approach. It helps to ensure a smooth transition while maintaining the desired therapeutic effect. These equivalent doses are suggested based on results from several studies and are illustrated in Table 3[34,69,71].

When switching from a drug administered once or twice daily such as liraglutide, oral semaglutide, or exenatide, we advise initiating the new product the day after discontinuing the original product. On the other hand, when switching from a drug administered weekly such as dulaglutide, semaglutide, exenatide extended release, or tirzepatide, we suggest beginning the new drug 7 d after discontinuing the original drug.

For patients who are tolerating the maximum therapeutic dose of a once-daily or twice-daily GLP-1 RA (exenatide 10 μg twice daily, liraglutide 1.8 mg once daily, or lixisenatide 20 mg once daily), but are switching to a once-weekly GLP-1 RA, we recommend starting dulaglutide or exenatide once-weekly at the maximum therapeutic dose (dulaglutide 1.5 mg and exenatide 2 mg) to decrease the HbA1C. For subcutaneous semaglutide and tirzepatide, we recommend starting at the intermediate once-weekly dose (semaglutide 0.5 mg and tirzepatide 5 mg) for 4 wk before transitioning to the maximum therapeutic dose. This approach can help minimize adverse GI events. However, when switching from 1 mg

Table 3 Suggested equivalent doses for different glucagon-like peptide-1 and dual glucose-dependent insulinotropic polypeptide/glucagon-like peptide-1 receptor agonists based on their impact on glycemic control

Agent	Route	Frequency	Equivalent dose									
Exenatide	SC	Twice daily	5 μg[1]	10 μg								
Lixisenatide	SC	Daily	10 μg[1]	20 μg								
Liraglutide	SC	Weekly	0.6 mg[1]	1.2 mg	1.8 mg							
Exenatide XR	SC	Weekly			2 mg							
Dulaglutide	SC	Weekly		0.75 mg[1]	1.5 mg	3 mg	4.5 mg					
Semaglutide	SC	Weekly		0.25 mg[1]	0.5 mg		1 mg	2 mg				
Semaglutide	PO	Daily	3 mg[1]	7 mg	14 mg							
Tirzepatide	SC	Weekly			2.5 mg[1]			5 mg	7.5 mg	10 mg	12.5 mg	15 mg

[1]The comparative efficacy of starting doses is not known and is based on the clinical experience of the authors in this group in various settings in different countries.
SC: Subcutaneous; PO: Oral.

semaglutide to tirzepatide, it is better to start tirzepatide with the 5 mg dose, as the HbA1C lowering effects of 5 mg tirzepatide and 1 mg semaglutide are similar. Later, the dose of tirzepatide can be increased to 7.5 mg and 10 mg after 4 wk[32]. For patients receiving subcutaneous once-weekly injections to be switched to once-daily oral semaglutide, manufacturers suggest initiating a 7 mg or 14 mg dose 7 d after their last injection. In contrast, patients receiving oral semaglutide 14 mg once daily can be switched to subcutaneous injection of semaglutide 0.5 mg, tirzepatide 5 mg, or dulaglutide 1.5 mg once-weekly the day after their last oral dose[26,72].

OTHER CONSIDERATIONS

Concerns over gastric stasis with GLP-1 and dual GLP-1/GIP RAs

The inhibitory effect on gastric motility and delayed gastric emptying seems to be a crucial factor contributing to the ability of GLP-1 RAs to reduce postprandial glycemia. GLP-1 RAs have demonstrated a dose-dependent deceleration of gastric emptying in both healthy and diabetic individuals; this effect applies to both the solid and liquid components of a meal[57]. This phenomenon is believed to be due to the rapid tachyphylaxis at the level of the vagal nerve activation[73,74]. However, the inhibitory effect of GLP-1RAs on gastric emptying might be diminished or absent in patients with diabetic-related dysautonomia[75].

Contrary to the prevailing expectation that long-acting GLP-1 RAs would lose their ability to slow gastric emptying with prolonged use, a study involving liraglutide revealed a persistent deceleration of gastric emptying, as assessed through scintigraphy, even after 16 wk of treatment. While the degree of deceleration was less pronounced than at the 5-wk mark, it remained significant[76]. Accordingly, it is now evident that both short- and long-acting GLP-1 RAs can continue to cause slow gastric emptying when used consistently, although short-acting GLP-1 RAs exhibit a more pronounced effect[57,73,77].

The reduction in postprandial glucose levels is closely associated with the extent of gastric emptying deceleration, as well as the baseline emptying rate. Importantly, baseline gastric emptying rates vary considerably among individuals, but this variability is less pronounced over time within individuals[26,57,69-73]. Hence, GLP-1 RA therapy could be considered for individuals with faster gastric emptying, which is often observed in cases of obesity and uncomplicated T2DM[76-79].

Preoperative management of patients on GLP-1 or dual GLP-1/GIP RAs

As discussed previously, GLP-1 RAs have been associated with several side effects such as nausea and vomiting which can be traced to their core mechanism of delaying gastric emptying. Recent findings from a case report and a retrospective study have shed light on significant clinical concerns[80,81]. They showed that patients exhibited residual gastric content even during the fasting period, which significantly increased the risk of pulmonary aspiration, with a causative factor attributed to the GLP-1 Ras[80,81]. Considering this evidence, The American Society of Anesthesiologists as part of preoperative management, recommends withholding all types of GLP-1 RAs before any elective surgery for 1 d before surgery on daily doses and 1 wk on weekly doses. Similarly, this can be extrapolated to dual GLP-1/GIP RA agents as well.

Missed doses

For the once-daily liraglutide, oral semaglutide, or twice-daily exenatide, patients may skip the missed dose and resume treatment with the next scheduled dose[23,24,26]. However, if a dose of lixisenatide is missed, the missed dose should be

administered 1 h prior to the next meal[25]. For the once-weekly exenatide or dulaglutide, the missed dose should be administered as soon as possible if there are ≥ 72 h until the next scheduled dose. If there are < 72 h before the next scheduled dose, the missed dose can be skipped and the next scheduled dose can be administered on time[21-23].

Regarding the injectable once-weekly semaglutide, administration is allowed within 5 d after the missed dose. However, if more than 5 d have passed, the dose can be skipped, and the next scheduled dose can be administered on time[26]. For tirzepatide, administration is done within 4 d after the missed dose. However, if more than 4 d have passed, the dose can be skipped, and the next scheduled dose can be administered on time[27].

Pregnancy and lactation

Generally, GLP-1 RAs are considered a category C drug in pregnancy due to reports of it being teratogenic in rat and rabbit controls; thus, their usage in pregnant women should be weighed against the risks of fetal complications[82]. Nevertheless, despite the paucity of research on their applicability and safety in humans during pregnancy, case reports have reported an uneventful pregnancy with the accidental usage of GLP-1 RAs during the first trimester of pregnancy [83,84]. However, the United States FDA advocates against the use of all GLP-1 and dual GLP-1/GIP RAs, including tirzepatide, for pregnant individuals and recommends the usage of contraception during the treatment period[24,26,27, 79]. Additionally, it is recommended to wait for at least 2 mo before planning pregnancy as a wash-out period in patients using injectable semaglutide[26]. It is important to note that the effectiveness of oral hormonal contraception may decline while a patient is on tirzepatide therapy[27,85]. Lastly, experience with the use of GLP-1 RAs in breastfeeding women is limited; therefore, their use is not recommended during lactation[85].

Use of GLP-1 or dual GLP-1/GIP RAs after bariatric surgery

Postprandial hyper-insulinemic hypoglycemia, a challenging metabolic phenomenon following bariatric surgery, continues to be a conflicting and stubborn complication to address. Continuous blood glucose monitoring indicates that it may happen in approximately 55% of individuals following laparoscopic vertical sleeve gastrectomy and up to 75% after Roux-en-Y gastric bypass (RYGB). Moreover, a significant number of these instances of hypoglycemia occur without any other accompanying symptoms. A recent systematic review indicated that GLP-1 RAs may potentially decrease the frequency of postprandial hypoglycemic episodes and enhance glycemic stability[86]. However, another study examining meal effect in humans after gastric bypass surgery showed that the augmented secretion and activity of GLP-1 play a significant role in post-meal hyperinsulinemia and altered glucose metabolism. This effect is particularly pronounced in individuals who experience hyperinsulinemia after gastric bypass surgery[87]. Another systemic review showed that following RYGB, individuals experiencing post-bariatric surgery hypoglycemia exhibit heightened GLP-1, insulin, and C-peptide in response to nutrients, with lower HbA1c levels. These findings propose that inhibiting GLP-1 could be a reasonable intervention to prevent hypoglycemia in patients dealing with post-bariatric surgery hypoglycemia following RYGB[88]. In addition, GLP-1 RAs showed a potential role in the remission of psoriasis, as noted in case reports following bariatric surgery, which opens up intriguing avenues for research and potential novel approaches to treating psoriasis [89].

In a recent review, semaglutide was deemed a viable treatment option for individuals experiencing weight regain following bariatric surgery[90]. On the other hand, no specific studies have been conducted to evaluate tirzepatide in post-bariatric surgery patients[91]. The initial data on the effectiveness of semaglutide in this patient group has recently emerged from a retrospective observational study conducted in Germany. This study involved patients who had either achieved inadequate weight loss or experienced weight regain after bariatric surgery. Following 6 mo of weekly subcutaneous administration of semaglutide at a maximum dosage of 0.5 mg, an average reduction in total body weight of 10.3% was observed[92].

In another retrospective observational study involving 50 patients who experienced weight regain after bariatric surgery, the effectiveness of the GLP-1 RAs liraglutide and semaglutide in reducing weight was investigated. The study found that after 6 mo of GLP-1 RA therapy, the median percentage of total body weight loss was 8.8%. Additionally, more than 75% of patients lost over 5% of their initial weight, and over 33% of them lost more than 10%. On average, patients had shed 67.4% of the weight that they had regained after their last bariatric procedure. Adverse events were recorded in roughly one-third of the patients, but they were all mild, temporary, and primarily related to the GI system. Overall, these findings endorse the safe use of both GLP-1 RAs for achieving clinically significant weight loss, amounting to roughly two-thirds of the weight gained after bariatric surgery[93].

Use of GLP-1 or dual GLP-1/GIP RAs in patients with renal insufficiency

Recently, there have been some post-marketing reports of acute renal injury or deterioration of pre-existing chronic kidney failure in patients treated with GLP-1 RAs. In certain cases, these conditions have necessitated the use of hemodialysis. It is worth noting that some of these events have been observed in patients without previously diagnosed kidney issues. The majority of the documented cases occurred in patients who had experienced nausea, vomiting, diarrhea, or dehydration during GLP-1 RA treatment. Therefore, it is important to monitor the kidney function in patients who are on GLP-1 RAs and complain of severe GI reactions[21-27].

It is not advisable to use GLP-1 RAs in patients with severe kidney dysfunction (creatinine clearance < 30 mL/min) or end-stage renal disease. Also, extreme caution is advised when prescribing GLP-1 RAs to patients with kidney transplantation or moderate kidney dysfunction (creatinine clearance 30-50 mL/min)[21-27].

The latest clinical trials have provided significant evidence of GLP-1 RAs demonstrating improvements in kidney function. One noteworthy trial is the LIRA-RENAL trial, which focused on evaluating the effectiveness and safety of liraglutide in diabetic patients with moderate kidney dysfunction (defined as eGFR 30-59 mL/min). This trial showed that adding liraglutide to the already existing glucose-lowering therapy significantly reduced HbA1C levels compared to

Table 4 Pharmacokinetic properties and renal outcomes of clinical trials with glucagon-like peptide-1 receptor agonists

Drug	Dose	Half-life (h)	Elimination	Clinical study	Renal benefit
Short-acting GLP-1 receptor agonists					
Exenatide	5-10 μg twice-daily SC	2.4	Mostly renal	None	None
Lixisenatide	10-20 μg once-daily SC	3.0	Mostly renal	ELIXA[65]	Lower rate of increase in urinary albumin-to-creatinine ratio
Long-acting GLP-1 receptor agonists					
Exenatide	2 mg QW SC	2.4	Mostly renal		
Liraglutide	0.6 mg, 1.2 mg or 1.8 mg once-daily SC	11.6-13.0	Peptidases and renal 6%; feces 5%	LEADER [64]	Nephropathy was decreased. UACR was decreased. RAS hormone was decreased. Progression to macroalbuminuria was decreased. Doubling of serum creatinine levels was decreased. eGFR of ≤ 45 mL/min per 1.73 m^2 was decreased. The initiation of renal replacement therapy was decreased. Risk of end-stage renal disease or renal death was decreased. Plasma renin concentration, renin activity, and angiotensin II were decreased
Semaglutide	0.5-1.0 mg once-weekly SC	165.0-184.0	Peptidases and renal	SUSTAIN-6[67]	Nephropathy > 35% was decreased. Progression to macroalbuminuria was decreased. Doubling of serum creatinine levels was decreased. eGFR of ≤ 45 mL/min per 1.73 m^2 was decreased. The initiation of renal replacement therapy decreased
Dulaglutide	0.75-1.5 mg once-weekly SC	About 112.8	Peptidases and renal	AWARD VII[66]	Reduced albuminuria, slower decline in renal function
Albiglutide	30-50 mg once-weekly SC	About 120.0	Peptidases and renal	None	None

GLP-1: Glucagon-like peptide-1; SC: Subcutaneous; UACR: Urinary albumin-to-creatinine ratio; RAS: Renin-angiotensin-system; eGFR: Estimated glomerular filtration rate; LEADER: Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results; SUSTAIN-6: Semaglutide Unabated Sustainability in Treatment of Type 2 Diabetes 6.

the placebo treatment (-1.05% *vs* -0.38%). Notably, the trial revealed no worsening in kidney function among the participants, and patients treated with liraglutide as compared to those who received the placebo showed a lower increase in albuminuria[94].

Another notable trial is the AWARD-7 trial which compared the benefits of the long-acting GLP-1 analog dulaglutide and insulin glargine on kidney function in patients with T2DM and moderate-to-severe chronic kidney disease. The trial involved 577 participants who were randomly assigned to three groups: Dulaglutide 1.5 mg, dulaglutide 0.75 mg, and insulin glargine. After 52 wk of treatment, the decline in the eGFR was significantly less in the dulaglutide-treated groups compared to the insulin group. This study confirmed the independent advantage of dulaglutide over glargine on the eGFR. Also, in the SUSTAIN-6 trial, patients receiving semaglutide had a lower rate of persistent macroalbuminuria compared to the placebo group[95]. The pharmacokinetic outcomes and renal benefits of clinical trials with GLP-1 and dual GLP-1/GIP RA agents are shown in Table 4[96].

Many pathophysiological mechanisms are attributed to the nephroprotective effect of GLP-1 RAs, one of which is the definite robust glycemic control achieved by the addition of GLP-1 RAs to the antidiabetic regimen of the patient which prevents the effect of high glucose concentration on increased filtration rate of proteins *via* the glomerular capillary membrane and on impaired tubular reabsorption[45]. Another possible theory is that it suppresses inflammation-related pathways, thus resulting in anti-inflammatory and antioxidative effects[97].

Use of GLP-1 or dual GLP-1/GIP RA during fasting

Fasting, as in the holy month of Ramadan, traditionally takes place from dawn to sunset. During this time, patients with diabetes mellitus may face some challenges in their daily doses and timing of medications. Different trials have demonstrated that the use of GLP-1 RAs during Ramadan is safe and effective with better glycemic control and weight reduction[98-102]. GLP-1 RAs have a potential hypoglycemia risk when used with other glucose-lowering agents[103]. Weekly injectable agents can be considered a good choice for fasting patients. It would be advisable to commence the weekly doses 4-8 wk before the month of Ramadan to closely monitor potential dehydration or GI upset to allow titration for tolerance[99]. Additional studies are required to determine the safety and effectiveness of tirzepatide and oral semaglutide during Ramadan. Based on previous data, liraglutide and lixisenatide were found to be safe, and therefore, GLP-1 RAs are unlikely to cause hypoglycemia during prolonged fasting. For example, exenatide was associated with a non-significant percentage of hypoglycemic events (0.08%), liraglutide was associated with fewer symptomatic hypoglycemia compared with sulfonylurea (P = 0.0009), lixisenatide and basal insulin led to fewer events compared to

sulfonylurea and basal insulin (odds ratio = 0.22; 95% confidence interval: 0.07-0.7)[98-100].

CONCLUSION

The utilization and switching between GLP-1 and dual GLP-1/GIP RAs pose complex challenges in various clinical scenarios, which have gained prominence with the increased availability of newer agents within these drug classes. While numerous studies have undertaken comparative evaluations among GLP-1 or dual GLP-1/GIP RAs, more studies are needed to examine the implications of switching between these emerging agents. This deficiency exposes a critical research gap, especially for healthcare professionals who are contemplating such transitions. Our aim is to accumulate clinical insights and offer practical guidance to healthcare practitioners navigating utilization and switching between GLP-1 or dual GLP-1/GIP RAs. This endeavor necessitates a comprehensive consideration of patient preferences, clinical variables, potential associated risks, and anticipated benefits.

FOOTNOTES

Author contributions: Alqifari SF, Alkomi O, and Esmail A contributed to the conceptualization of this study; Alqifari SF and Alkhawami K were involved in the methodology of this review; Yousri S and Muqresh MA participated in the validation of this manuscript; Alharbi N and Khojah AA took part in the formal analysis of this article; Khojah AA and Aljabri A curated the data; Alqifari SF, Alkomi O, Esmail A, Allahham A, Prabahar K, and Alshareef H contributed to the writing-original draft preparation; Alqifari SF, Alkhawami K, Alrabiah A, and AlBishi LA were involved in the writing-review and editing; Alqifari SF, Alharbi N, and AlBishi LA took part in the visualization of this study; Alqifari SF contributed to the supervision; Alqifari SF and Alkomi O were major in the project administration; Alqifari SF and Alrabiah A contributed to the funding acquisition; and all authors have read and agreed to the published version of the manuscript.

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REVIEW

Association of autoimmune thyroid disease with type 1 diabetes mellitus and its ultrasonic diagnosis and management

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Abstract

As a common hyperglycemic disease, type 1 diabetes mellitus (T1DM) is a complicated disorder that requires a lifelong insulin supply due to the immune-mediated destruction of pancreatic β cells. Although it is an organ-specific autoimmune disorder, T1DM is often associated with multiple other autoimmune disorders. The most prevalent concomitant autoimmune disorder occurring in T1DM is autoimmune thyroid disease (AITD), which mainly exhibits two extremes of phenotypes: hyperthyroidism [Graves' disease (GD)] and hypothyroidism [Hashimoto's thyroiditis, (HT)]. However, the presence of comorbid AITD may negatively affect metabolic management in T1DM patients and thereby may increase the risk for potential diabetes-related complications. Thus, routine screening of thyroid function has been recommended when T1DM is diagnosed. Here, first, we summarize current knowledge regarding the etiology and pathogenesis mechanisms of both diseases. Subsequently, an updated review of the association between T1DM and AITD is offered. Finally, we provide a relatively detailed review focusing on the application of thyroid ultrasonography in diagnosing and managing HT and GD, suggesting its critical role in the timely and accurate diagnosis of AITD in T1DM.

Key Words: Type 1 diabetes mellitus; Autoimmunity; Autoimmune thyroid disease; Ultrasonography; Diagnosis

Core Tip: Although type 1 diabetes mellitus (T1DM) is an organ-specific autoimmune disease, patients with this disease are more prone to develop other autoimmune disorder, and the most prevalent autoimmune disorder in T1DM patients is autoimmune thyroid disease (AITD). Undiagnosed and untreated AITD may lead to metabolic disturbances and impair diabetes care in T1DM patients, warranting regular and long-term observation. We herein offer an updated review of the basic characteristics of both diseases and factors contribute to their concomitant presence. Additionally, we focus on the role of thyroid ultrasonography in the diagnosis and management of AITD.

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INTRODUCTION

As a common childhood-onset chronic disorder, type 1 diabetes mellitus (T1DM) affects 1:300 children, and the disease incidence has continued to increase in recent decades[1,2]. The incidence of T1DM is not uniform across the world, and it tends to be higher in higher-income countries than in lower-income countries[3]. As a result of the autoimmune attack predominantly driven by T cells, T1DM occurs in genetically predisposed individuals exposed to environmental and stochastic factors, leading to the dysfunction and death of pancreatic β-cells, with subsequent hyperglycemia[4]. However, although T1DM is an organ-specific autoimmune disorder, individuals with T1DM often exhibit a higher risk of additional autoimmune disorders[5]. The concomitant presentation of T1DM and another autoimmune disorder may complicate diabetes management and result in varying clinical symptoms, thus seriously influencing patient quality of life[6]. Among these additional autoimmune disorders co-occurring among children and adolescents with T1DM, autoimmune thyroid disease (AITD) accounts for the highest proportion[7]. As the most prevalent organ-specific immune-mediated disorder in the world, AITD is characterized by autoreactive lymphocyte infiltration in the thyroid and the presence of autoantibodies targeting thyroid antigens[8]. Clinically, thyroid dysfunctions, which include hyperthyroidism and hypothyroidism[9], lead to metabolic disturbances and may impair diabetes management in T1DM. Therefore, it is important for individuals with T1DM to regularly screen for thyroid disorders, allowing for the early detection, early diagnosis and intervention of thyroid dysfunction. Benefiting from advances in ultrasound technology, ultrasonography has been widely used to evaluate and treat thyroid diseases[10]. Here, we aim to provide an updated review about the relationship between T1DM and AITD as well as the current status of ultrasonography application in AITD.

THE BASIC CHARACTERISTICS OF T1DM AND AITD

T1DM

As described above, the estimated incidence of T1DM is increasing in many areas around the world. However, these incidences of T1DM may be underestimated. These numbers do not include many adults with T1DM, as almost all incidence data are derived from registered individuals under 20 years of age[11]. Additionally, there is a clear male predominance in T1DM individuals, and this may be associated with the protective role of estrogen[12].

Although the etiology and pathogenesis mechanisms of T1DM have many unknow and large knowledge gaps, our understanding of its pathological process has greatly improved during the last two decades. It has been suggested that a complicated interaction among genetic, environmental, and immunologic factors induces a T-cell-regulated immune attack directed against pancreatic β cells[4,13]. Genetic studies have revealed that T1DM genetic susceptibility exhibits a polygenic nature. Currently, there are more than 60 gene loci linked to T1DM[4]. Among them, the human leukocyte antigen (HLA) class II genes involved in antigen presentation exhibit a major risk factor for T1DM, and HLA-DR and HLA-DQ show the strongest relationship with this disease[14]. In addition, various other immune-related loci (non-HLA) connected to T1DM are recognized, such as *CTLA4* (cytotoxic T-lymphocyte antigen 4) and *PTPN22* (protein tyrosine phosphatase non-receptor type 22). Furthermore, various candidate genes as well as noncoding RNAs have been identified based on genome-wide association studies (GWAS)[15]. This strong genetic component of T1DM has stimulated efforts to develop a T1DM genetic risk score based on single-nucleotide polymorphism genotyping, as it would be useful for evaluating and predicting islet autoimmunity progression as well as T1DM development in high-risk individuals[16].

However, compared to genetic factors, environmental influences remain poorly understood despite intensive research. The increasing incidence, twin studies, and immigrant studies indicate that environmental factors also exhibit a major role in contributing to T1DM development[17]. Numerous research findings have indicated that the environmental triggers connected to T1DM mainly include climatic conditions, diet, lifestyle, obesity, toxins, vitamin D sufficiency, and infections[17,18]. All these factors may lead to gut microbiota dysbiosis and influence the interrelationship between the intestinal microbiota and host immune system, potentially contributing to T1DM[19,20]. However, some research results

related to the role of various environmental factors are largely controversial[21,22], and this may reflect the heterogeneity of T1DM. Therefore, further work concerning the role of gene-environment interactions in contributing to T1DM development is needed.

Influenced by potential genetic and environmental factors, β cell-directed autoimmunity, which includes humoral and cell-mediated autoimmunity, is triggered during the initiation of the development of T1DM. Before clinical symptoms present, there is a long preclinical stage, characterized by the production of disease-specific autoantibodies and reduced insulin and C-peptide production and secretion. The best-characterized autoantibodies connected to T1DM are those that recognize islet cells, insulin, glutamic acid decarboxylase 65, islet tyrosine phosphatase 2, and zinc transporter 8[23]. These nonpathogenic autoantibodies can be viewed as biomarkers of the autoimmune process. Therefore, according to the appearance of autoantibody(ies) and clinical manifestations, a disease staging classification system has been introduced to evaluate and predict T1DM progression in genetically at-risk individuals[24]. Three stages have been defined, starting from serological autoimmunity (≥ 2 disease-related autoantibodies with normoglycemia, stage 1) to a second stage of dysglycemia (stage 2), and to definitive diagnosis of T1DM (stage 3). As it is important to guide the predication and prevention of T1DM, this classification scheme should be further revised by identifying novel stage-specific biomarkers[25,26]. In nonobese diabetic mice, both $CD4^+$ and $CD8^+$ T cells contribute to T1DM development, and in individuals with T1DM, T cells targeting T1DM-related autoantigens can be observed in the pancreatic lymph nodes and islets[27,28]. The participation of these potentially pathogenic T cells in the immune attack toward β-cells suggests the failure of immune system regulation. Of note, the gatekeeper role of regulatory T cells (Tregs) is important to maintain immunological tolerance and prevent autoimmune disease[29]. Thus, a reduction in different Treg populations, especially $CD4^+CD25^+Foxp3^+$ Tregs, contributes to the development of T1DM[30]. As a result, various immune cells infiltrate the islets, resulting in insulitis. Insulitis is an early pathologic hallmark of this autoimmune disorder and eventually causes the death of β-cells and a reduction in insulin[31]. In addition, it has been proposed that β-cells are not merely passive targets of autoimmune reactions but also contribute to the initiation of this complex autoimmune process[32,33].

At present, there are no widely accepted and validated diagnostic criteria for T1DM. Instead, its clinical diagnosis still mainly depends on two main features, including insulin deficiency as well as the presence of the corresponding autoantibodies. However, additional criteria are needed as the diagnostic accuracy of the above criteria in individuals who develop diabetes over the age of 20 years is less informative[34]. Once diagnosed, individuals with T1DM must rely on exogenous insulin for glycemic control to avoid ketoacidosis and hyperglycemia-related complications[35]. However, insulin therapy does not represent a cure and often fails to achieve optimal blood sugar management in many patients. Based on the understanding of its heterogeneity and early-stage development as described above, more personalized medicine approaches should be designed to diagnose, prevent, and hopefully treat T1DM[36,37]. However, as an autoimmune disease, the ultimate optimal goal of T1DM treatment is to restore immune tolerance toward disease-specific autoantigens to avoid autoimmune attack against β-cells. For this purpose, combination therapy based on antigen-specific immunotherapy exhibits promising prospects[38,39].

AITD

As the most prevalent organ-specific autoimmune disorder all over the world and the most prevalent pathological condition associated with the thyroid gland, AITD affects approximately 5% of the total world population[40]. Graves' disease (GD) and Hashimoto's thyroiditis (HT) represent its two main clinical manifestations. The incidence of HT in females and males is approximately 3.5/1000 and 0.6/1000, respectively, with a global prevalence of 2% to 3%. GD influences 1% to 2% of females and 0.1% to 0.2% of males[40]. In contrast to the male predominance in T1DM, AITD shows a strong female preponderance, which may result from the immune-enhancing activity provided by estrogenic sex steroids[41]. Thus, the reasons behind these sex differences in these autoimmune diseases deserve more attention and research in the future.

As a result of immune imbalance, tolerance toward thyroid-specific autoantigens, such as thyroglobulin (Tg), thyroperoxidase (TPO) as well as thyroid-stimulating hormone receptor (TSHR), lost, leading to an immune destruction of thyroid tissue, yielding AITD[40]. Autoreactive T and B lymphocyte infiltrates within the thyroid and the presence of antibodies targeting the above thyroid self-antigens (anti-Tg, anti-TPO, and anti-TSHR antibodies) can directly confirm that autoimmune reactions occurr in both GD and HT. Compared to those in GD, lymphocyte infiltrates in HT are more severe, and therefore, HT patients exhibit the destruction of thyroid follicles, leading to low thyroid function (hypothyroidism)[42]. However, as the production of TSHR-specific stimulating antibodies (TSAbs) is redundant in GD, thyrocyte proliferation, thyroid growth, and the production of thyroid hormones are induced, finally inducing hyperthyroidism[43]. Both diseases exhibit different clinical manifestations. However, HT and GD share similar immunogenetic mechanisms, and conversion between conditions can occur[44,45].

During the last two decades, major progress on the mechanisms underlying the development of AITD has been made based on extensive research. Generally, it is believed that a complicated interaction between genetic susceptibility and environmental risk factors, together with various epigenetic factors, contributes to the pathogenesis of AITD[40,42,43]. Among these factors, genetic factors predominate, as they account for 70% to 80% of the risk of developing thyroid autoimmunity based on twin/family studies. Environmental factors account for the remaining 20% to 30%[46,47]. The identification of genes associated with AITD susceptibility has contributed to a better understanding of disease-causing mechanisms and has indicated that the presence of the related genes exacerbates AITD risk[48]. The main known AITD susceptibility genes can be mechanistically divided into general immune-regulatory genes (such as *HLA-DR3*, *CTLA-4*, and *PTPN22*) as well as thyroid-specific genes, such as the genes encoding the corresponding autoantigens (*Tg*, *TPO*, and *TSHR*). In addition, various novel candidate risk genes for AITD, such as *FCRL3* (FCReceptor-Like-3), *SCGB3A2* (secretoglobin 3A2), and *TNFR 2* (tumor necrosis factor receptor 2), have been described by GWAS and immunochip analysis[40,49]. As genetic factors play a major role in triggering AITD, individuals with family members who develop this disease

exhibit a high risk of AITD. Therefore, to get a precise answer to the question asked by individuals with AITD "Will my daughter or my sister also get this disorder?", the Thyroid Hormones Event Amsterdam (THEA) score was designed and applied for predicting AITD risk in healthy female subjects who had at least one relative with AITD based on the various baseline characteristics. This THEA score performs accurately and seems to be useful for young women of AITD families [50]. However, this THEA score still needs to be further validated externally.

In addition, for a given genetic risk factor in AITD, epigenetic modifications mediated by DNA methylation[51], histone modifications[52], and noncoding RNAs[53] may be necessary to trigger AITD. However, the promoting mechanism of such epigenetic modifications in AITD have not been fully elucidated, and therefore, more research should be done to further investigate their roles in AITD pathogenesis and to develop better diagnostic, prognostic, and therapeutic tools. Some environmental factors may induce corresponding epigenetic modifications, and subsequently trigger AITD in genetically susceptible individuals, indicating that epigenetic modifications seem to narrow this gap between genetic and environmental factors[54,55]. Several AITD-related environmental factors have been confirmed, such as iodine status, smoking, alcohol intake, selenium supplementation, vitamin D deficiency, infections, stress, and drugs[47]. Thus, preventive interventions, namely, the modulation of exposure to particular environmental risk factors, may diminish the corresponding risk in individuals at risk for developing AITD. However, there are few effective preventive interventions to diminish this risk, and these few options are not always feasible[47].

As a result of the interaction between the above various factors, the balance of immune homeostasis is disrupted, inducing a loss of tolerance toward thyroid-specific autoantigens and finally the onset of AITD[56]. Effector T cells and their secreted cytokines contribute greatly to the pathogenic development of HT and GD[57,58]. Traditionally, Th1/Th2 cell imbalance is viewed as the main driver of autoimmunity in AITD. Th1 cells may induce apoptotic pathways in thyroid follicular cells by secreting IFN-γ and IL-2, resulting in the destruction of thyroid cells. Th2 cells, which mainly produce IL-4, IL-5, and IL-13, may induce thyroid growth and overactivity by enhancing TSAbs release[59,60]. In addition, numerous recent studies have demonstrated the pathogenic functions of IL-17 and Th17 cells and Th17/Treg imbalance in both HT and GD[61]. This is important for future research to discover Th17-related therapeutic targets.

Accurately diagnosing GD or HT is important, and this mainly relies on the measurement of serum levels of thyroid stimulating hormone, free thyroid hormones (FT3, FT4) as well as the corresponding autoantibody levels. In addition, cytological examination, thyroid ultrasonography, and radiological evaluation may be needed in some cases[62,63]. If a definitive diagnosis was established, the most appropriate patient management decision could be made. For GD treatment, mainly including thyroidectomy, radioiodine therapy, antithyroid drugs, and β-blockers, there have been no major changes in recent years[62]. For HT treatment, oral administration of a synthetic hormone is used to control hypothyroidism. In addition, diet management is advised[63]. Although these available treatments are effective for HT and GD, there are still some limitations. Thyroid hormone substitution therapy in HT does not target the disease process [64]. Available treatments performed in GD may have the potential to cause some side effects[62,65]. Therefore, the clinical management of AITD remains an active area that requires further investigation, especially by improving understanding of its pathophysiology to discover therapeutic approaches targeting the underlying autoimmune process.

THE CONCOMITANT PRESENCE OF T1DM AND AITD

The occurrence of one autoimmune disorder enhances the risk for the development of others. Therefore, the coexistence of two or more autoimmune endocrinopathies is termed autoimmune polyendocrine syndrome (APS). However, sometimes there may be additional (non)glandular autoimmune disease(s) present[66]. There are two major types of APS, including juvenile type I and adult APS with three variants or subtypes (type II to IV)[66,67]. An economic evaluation of the costs for patients with APS in Germany has shown that T1DM is the main cost driver in APS[68]. APS type III, encompassing T1DM and AITD (HT or GD), is the most prevalent APS type, and it can often be associated with other (non)glandular autoimmune disorders, excluding Addison's disease[69,70]. Various studies have observed a higher rate of thyroid disorder among T1DM patients compared with the general population, suggesting that AITD represents the most prevalent autoimmune disorder concomitant with T1DM[5,71,72]. Existing data show that approximately one-third of T1DM individuals develop AITD within a few years, and this proportion increases up to 50% in anti-TPO autoantibody-positive T1DM individuals. Additionally, the incidence of HT among T1DM individuals is relatively higher than that of GD[73,74]. Conversely, the prevalence of T1DM is also enhanced in patients with HT or GD, and the incidence of T1DM in HT individuals is relatively higher than that in GD individuals[75,76].

As described above, both T1DM and AITD are common organ-specific autoimmune disorders, and a complicated interaction between genetic factors and environmental stimuli, together with various immune events or epigenetic factors, induces the autoimmune process to destroy the target tissue (the β-cells in T1DM and the thyroid in AITD; Figure 1). While differences in the pathogenesis responsible for both disorders persist, the relatively high concomitant presence rate of T1DM and AITD in the same individual or family indicates that these two diseases may share pathogenic factors within the induction of the corresponding autoimmune process[77]. Various genes have been confirmed to contribute to the risk of both T1DM and AITD; these are referred to as joint susceptibility genes for APS type III (Figure 1) [73,77-79]. Among these susceptibility genes, *HLA* genes remain the most important contributor[73,77]. Based on the interaction with susceptibility genes, environmental factors are necessary to trigger autoimmune responses in both T1DM and AITD. It has been shown that infection (such as *Helicobacter pylori* infection), vitamin D deficiency, as well as multiple chemokine (C-X-C motif) ligands could confer susceptibility to both diseases[77]. Therefore, the combined influence of these susceptibility risk factors may stimulate the corresponding autoimmune processes in various organs of the same individual or in families (Figure 1). As there may be a rather long time interval between the first occurrence of one

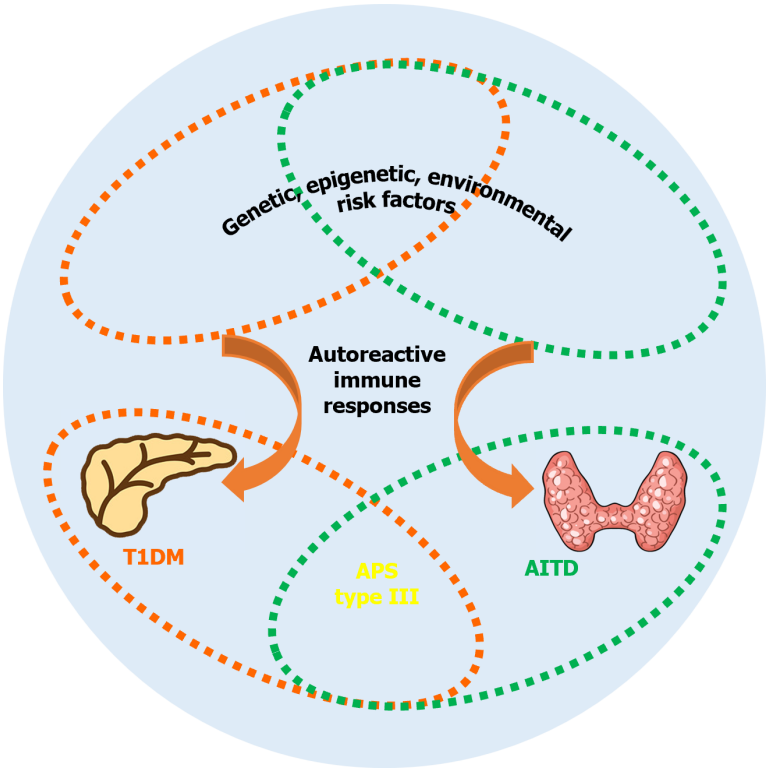

Figure 1 The concomitant presence of type 1 diabetes mellitus and autoimmune thyroid disease. Type 1 diabetes mellitus and autoimmune thyroid disease may share pathogenic risk factors within the induction of the corresponding autoreactive immune responses. T1DM: Type 1 diabetes mellitus; AITD: Autoimmune thyroid disease; APS: Autoimmune polyendocrine syndrome.

autoimmune endocrinopathy and the other, long-term monitoring and regular evaluation of patients and their relatives is warranted, such as the detection of associated autoantibodies[80] and thyroid ultrasound[81].

ULTRASONOGRAPHY APPLICATION IN AITD

As it is noninvasive without known detrimental bioeffects and affordable, ultrasound has been widely applied in the clinic for decades. Low-resolution B-mode ultrasound was first introduced for thyroid imaging in 1967[82], and ultrasonography is currently considered crucial in the diagnosis and management of thyroid disorders, including AITD [81,83].

Ultrasonography in HT

As mentioned above, the cellular and humoral immunity involved in the development of HT results in morphologic and microscopic changes in thyroid tissue, such as thyroid enlargement, lymphoplasmacytic infiltration, fibroplastic proliferation, lymphatic follicular formation, calcification, vascular proliferation, and parenchymal atrophy[63]. These changes influence the ultrasonographic characteristics of HT. Generally, a moderate grayscale uniform echo image, with a higher signal compared to the surrounding muscles, can be observed in the structurally normal thyroid. As a result of thyroid infiltration in HT, a heterogeneously hypoechoic thyroid can be observed, and thus, this hypoechogenicity can be used for clarifying diagnosis[84,85]. In addition, pseudonodules and inhomogeneous parenchyma can also be observed, which could be due to fibroplastic proliferation[86].

However, the sonographic appearances detected in HT vary greatly and may be indistinguishable from other thyroid disorders[87,88]. Therefore, in some atypical cases, multiple sonographic characteristics obtained from various ultrasound imaging technologies should be considered. The vascularity type of "focal inferno" observed by color Doppler ultrasound is a characteristic of focal HT, which is a special form of HT, and this is crucial to determine the corresponding treatment strategy[89]. In anti-TPO autoantibody-positive euthyroid subjects, comprehensive parameters obtained by ultrasound and power Doppler ultrasound exhibited a diagnostic accuracy of 87.2%, sensitivity of 90%, specificity of 84.8%, negative predictive value (NPV) of 90.7%, and positive predictive value (PPV) of 83.7% for the diagnosis of HT[90]. The cutoff value for thyroid tissue elasticity obtained from real-time ultrasound elastography for diagnosing HT showed 96% sensitivity and 67% specificity in adults[91], as well as 97.4% sensitivity and 100% specificity in children[92]. Based on ultrasound 2D shear-wave elastography, thyroid stiffness measured by shear-wave dispersion performed somewhat better in diagnosing HT than thyroid viscosity measured by shear-wave dispersion[93]. Compared with conventional ultrasound examination, high-frequency ultrasonic elastography exhibited a significantly higher diagnostic accuracy of HT (sensitivity, 92.16%; specificity, 92.86%; NPV, 86.67%; PPV, 95.92%)[94]. A recent meta-analysis indicated that

ultrasound-based shear wave elastography plays an important role and should be encouraged for use in diagnosing pediatric HT[95].

In addition, ultrasound acquisition and interpretation are highly subjective and somewhat operator dependent, even irreproducible in some cases[96]. To avoid subjective differences, a computer-assisted diagnostic system based on feature extraction and classification as well as a machine learning algorithm was proposed to provide objective and reproducible interpretation results in the diagnosis of HT, yielding a diagnostic accuracy of 80%[97], 85%[98], and 79%[99]. Recently, artificial intelligence (AI)-aided diagnosis of thyroid disorders has attracted growing interest[100,101]. A convolutional neural network-based computer-aided HT diagnostic system was evaluated and validated in a large number of samples, including 39280 ultrasonic images from 21110 individuals. The results show that this strategy significantly improved the radiologists' diagnostic efficiency of HT, as it exhibited high performance (89.2% accuracy, 89% sensitivity, and 89.5% specificity)[102]. A later report in 2022 developed a deep learning-based diagnostic system for HT (HTNet) through training and testing in a larger number of samples, and HTNet significantly exceeded the performance of radiologists in terms of accuracy and sensitivity. The corresponding diagnostic performance of HTNet can be further improved by integrating serologic markers[103]. Therefore, these computer-assisted ultrasound diagnostic systems based on novel AI show promising prospects in HT management and thus could be tested in prospective clinical trials.

Cervical lymph nodes (CLNs) are often observed in HT patients[104]. Fine needle aspiration biopsy (FNAB), an invasive intervention, has been regarded as the gold standard to diagnose, differentiate, and recognize CLNs as true nodules or pseudonodules[105]. To avoid the use of unnecessary invasive biopsies, sonoelastography should be applied, as it can detect true thyroid nodules (TNs) with a similar accuracy and sensitivity to FNAB[106]. An enhanced number of enlarged CLNs without a significant increase in lymph node size was observed on the sonographic images of HT patients [107], and an enhanced frequency of CLNs with abnormal ultrasonographic characteristics has been observed in HT patients[108]. Therefore, further understanding of the sonographic characteristics of CLNs in HT patients may be useful to improve the diagnosis of HT and avoid unnecessary invasive tests.

In addition, TNs can be frequently detected among HT patients, and these nodules often exhibit poor uptake of radioisotopes, indicating the possibility of malignancy and suggesting a possible association between HT and thyroid cancer[109,110]. However, whether HT increases thyroid cancer risk in individuals with TNs is controversial and remains to be defined[111,112]. Therefore, to avoid overtreatment with surgery in HT patients with TNs without any other evidence of malignancy as well as to predict the malignancy risk of these TNs accurately, various ultrasound-based diagnostic classification systems, which have been developed for differentiating benign and malignant TNs, may represent a critical role in detecting malignant TNs in HT individuals[113-116]. Moreover, in some cases with difficult diagnoses, ultrasound-guided FNAB can be used as an effective, less-invasive approach to confirm the nature of the lesion and propose the most beneficial/optimal treatment[117,118]. For the treatment of benign TNs in HT patients, ultrasound-guided microwave ablation shows a promising trend[119].

Ultrasonography in GD

As described above, autoantibodies against TSHR (TSAbs) drive GD pathogenesis. However, the role of TSAbs in GD is different from that of autoantibodies causing tissue damage in many other autoimmune disorders. TSAbs stimulate the thyroid and increase the production and secretion of thyroid hormones, therefore causing goiter and hyperthyroidism [62]. Apart from clinical presentations and laboratory findings, Doppler ultrasound measuring thyroid blood flow is widely applied in diagnosing GD[120]. However, it should be noted that the application of ultrasound in GD management, which mainly focuses on academic interest, has not gained much clinical importance thus far compared with that of some other thyroid disorders, such as thyroid cancer[81,121].

Features of an increased thyroid gland volume, diffusely low thyroid echogenicity as well as hypervascularity have been shown in GD[121,122]. At variance with the hypoechogenicity resulting from diffuse lymphocytic infiltration in HT as described above, the hypoechogenic pattern observed in GD may result from decreased colloid content with enhanced cellularity and a decrease in the cell-colloid interface and/or from enhanced blood flow[122,123]. Alternatively, it can be said that hypoechogenicity is not specific for HT, as it can also be observed in GD. Therefore, it has been shown that conventional grayscale ultrasound exhibits a high specificity with low sensitivity in diagnosing and differentiating GD and HT, and it is difficult to differentiate between both disorders using conventional grayscale ultrasound alone as a result of those significant overlaps in ultrasonographic images[124].

During the late 20th/early 21st centuries, Doppler ultrasonography, including color Doppler and power Doppler, has been widely studied to diagnose, evaluate, and manage GD, and the characteristic intense Doppler flow referred to as the "thyroid inferno" pattern has been well defined in this disease, yielding a high specificity in differentiating GD from other triggers of hyperthyroidism[125-130]. However, at that time, little effort was made to emphasize the role of Doppler ultrasonography in GD, leading to its underutilization in diagnosing this thyroid disorder. Therefore, a call to include an ultrasound protocol with Doppler patterns in the clinical diagnosis of GD was raised in 2009[131]. Since then, various methods based on Doppler ultrasonography have been widely and further investigated for their roles in GD management. The diagnostic utility of the peak systolic and/or end-diastolic velocities (PSV and EDV) in the superior and/or inferior thyroid artery measured by color Doppler ultrasonography is comparable to the performance of TSAb and Tc-99m pertechnetate uptake to differentiate GD from painless (or silent) thyroiditis[132,133]. Compared to EDV, PSV is a more useful parameter in differentiating GD from HT[88]. Although thyroid ultrasound is less accurate than both autoantibody immunoassays and thyroid scintigraphy in diagnostic testing for Graves' or non-Graves' hyperthyroidism, the "thyroid inferno" pattern shows a high PPV toward GD[134]. However, as these methods are highly operator-dependent and subjective, the interobserver variability as well as the difficulty in quantifying the corresponding results objectively remain their major limitations. Therefore, a newly developed analysis software that can quantify color Doppler signals, entitled "Color Quantification" (CQ), has been introduced. The results show that the increased CQ

values help diagnose GD, and therefore, the CQ technique exhibits promise in diagnosing GD[135]. In addition, a new-generation Doppler designed for improving diagnostic sensitivity, microvascular ultrasonography, has also been tested regarding its ability in the differential diagnosis of GD and HT[136] or destructive thyroiditis[137] in a quantitative and real-time manner with low intra- or inter-observer variability. In addition, some tests analyzing the ability of shear-wave elastography in diagnosing GD show that it can be applied as a complementary technique to facilitate the diagnosis of GD[138] or the differential diagnosis of GD and HT[139].

Apart from the diagnosis or differential diagnosis of GD, ultrasound contributes a lot to treat and manage this thyroid disorder. The sonographic appearance of the thyroid gland can be used to classify GD into different clinical courses and autoimmune activities[140-142]. Therefore, color pixel density calculated based on the color-flow maps obtained with color duplex ultrasonography can be used to evaluate the optimal dose of antithyroid drugs to maintain euthyroid status in GD[143]. In addition, it is important to predict outcome in GD patients after drug withdrawal. Thus, color Doppler ultrasonography may be a useful tool to detect a relapsing course of hyperthyroidism and, therefore, facilitate the offering of an adequate therapeutic approach[126,128]. As described above, thyroidectomy may need to be performed in some GD patients, and preoperative color Doppler sonography evaluating the superior thyroid artery may be useful to identify those individuals who are more prone to bleeding intraoperatively[144]. Concurrent differentiated thyroid cancer occurs in pediatric GD patients, and it has been suggested that ultrasound examination should be included for those with an abnormal thyroid at palpation to select patients for appropriate definitive therapy, such as thyroidectomy[145,146]. In addition, surgery and radioactive iodine (RAI) therapy are recommended for individuals with persistent/relapsed GD [147]. However, many patients may not want to accept surgery or RAI therapy as a result of the possible risks from surgery and radiation[148,149]. Therefore, one preliminary study applied and evaluated ultrasound-guided high-intensity focused ultrasound ablation as a novel manner to treat medically refractory GD, and the results show that this strategy may be a safe and efficacious method for treating persistent/relapsed GD[150]. This usefulness was confirmed based on the outcomes (specifically, disease relapse and safety) over the two years of follow-up[151].

CONCLUSION

T1DM and AITD (HT and GD) represent the two most frequent autoimmune endocrine disorders. Accumulating evidence indicates that T1DM and AITD share similar immunogenetic susceptibilities; therefore, both diseases often cluster in individuals as well as families. AITD has been the most prevalent comorbid autoimmune disease of T1DM. Thus, a timely and accurate diagnosis of AITD in T1DM patients is particularly crucial for diabetes management. For this purpose, thyroid ultrasonography exhibits a critical role in the diagnosis and management of AITD.

FOOTNOTES

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REVIEW

Metabolic disorders in prediabetes: From mechanisms to therapeutic management

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Abstract

Diabetes, one of the world's top ten diseases, is known for its high mortality and complication rates and low cure rate. Prediabetes precedes the onset of diabetes, during which effective treatment can reduce diabetes risk. Prediabetes risk factors include high-calorie and high-fat diets, sedentary lifestyles, and stress. Consequences may include considerable damage to vital organs, including the retina, liver, and kidneys. Interventions for treating prediabetes include a healthy lifestyle diet and pharmacological treatments. However, while these options are effective in the short term, they may fail due to the difficulty of long-term implementation. Medications may also be used to treat prediabetes. This review examines prediabetic treatments, particularly metformin, glucagon-like peptide-1 receptor agonists, sodium glucose cotransporter 2 inhibitors, vitamin D, and herbal medicines. Given the remarkable impact of prediabetes on the progression of diabetes mellitus, it is crucial to intervene promptly and effectively to regulate prediabetes. However, the current body of research on prediabetes is limited, and there is considerable confusion surrounding clinically relevant medications. This paper aims to provide a comprehensive summary of the pathogenesis of pre-diabetes mellitus and its associated therapeutic drugs. The ultimate goal is to facilitate the clinical utilization of medications and achieve efficient and timely control of diabetes mellitus.

Key Words: Prediabetes; Glucagon-like peptide agonists; Sodium–glucose cotransporter 2 inhibitors; Vitamin D; Chinese herbal medicines

Core Tip: Addressing the global impact of diabetes, this review underscores the pivotal role of pre-diabetes as a precursor and the window of opportunity it offers for reducing diabetes risk. While interventions like lifestyle changes and pharmacological treatments prove effective in the short term, sustained implementation remains challenging. The review delves into the potential of medications, including metformin and other agents, shedding light on the current limitations in research and clinical confusion. By providing a comprehensive overview, the paper aims to enhance understanding, enabling more efficient and timely control of diabetes mellitus.

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INTRODUCTION

Prediabetes, also known as impaired fasting glucose or impaired glucose tolerance (IGT), is a condition that has affected approximately 213000 young individuals in the United States as of 2017, with an estimated 239000 individuals projected to be affected by 2060, based on current growth trends[1]. Timely management of prediabetes can reduce the incidence of diabetes, particularly type 2 diabetes[2]. Prediabetes refers to blood glucose levels that are higher than normal but below the glucose levels detected in patients with diabetes[3,4]. Moreover, individuals with prediabetes are in a sub-healthy state, somewhere between being healthy and clinically diabetic. Effective treatment of prediabetes can prevent the development of diabetes and help individuals to return to a healthy state.

Interventional therapy for prediabetes primarily includes lifestyle interventions and pharmacological treatments. Commonly used clinical drugs include metformin, glucagon-like peptide (GLP-1) agonists, sodium–glucose cotransporter 2 (SGLT2) inhibitors, vitamin D supplements, and Chinese herbal medicines. In this review, we provide an overview of prediabetes and its therapeutic agents. The ultimate aim of this article is to offer insights into prediabetes and contribute to the development of effective treatment strategies.

PREDIABETES

Prediabetic contributory factors may include genetics and diets high in calories and fat[5]. Such diets contribute to excess fat accumulation and compensatory lipolysis within the body, resulting in an increased free fatty acid (FFA) content. The FFAs can disrupt cellular homeostasis, hinder cellular insulin response, reduce cellular uptake and utilization, increase the risk of insulin resistance in the liver, and damage muscles and the liver, ultimately leading to the development of diabetes[6].

The criteria for diagnosing prediabetes include a fasting plasma glucose level of 100-125 mg/dL (5.6-6.9 mmol/L), a 2-h oral glucose tolerance test (OGTT; 75 g 2 h) result of 140-199 mg/dL (7.8-11.0 mmol/L), and a glycated hemoglobin (HbA1c) level of 5.7%-6.4% (39-47 mmol/mol)[7]. It is important to note that, among these criteria, the HbA1c test is only applicable to adults. IGT is a key diagnostic criterion for prediabetes; however, HbA1c and fasting blood glucose (FBG) levels are also used in the diagnosis, as shown in Table 1[8-11].

A clinical survey in the United States reported that the prevalence of prediabetes is as high as 30%, indicating that approximately one in three adults has a fasting glycemic index or HbA1c level that meets the criteria for prediabetes[12]. Meanwhile, in India, the number of individuals with IGT reached 25.2 million in 2019 and is expected to reach 35.7 million by 2045[13]. The prevalence of prediabetes has notably increased from 15.5% in 2008 to 38.1% in 2018 in China (Table 2)[14-16].

Patients with prediabetes may exhibit characteristics associated with diabetes, including weight and blood glucose abnormalities and systemic insulin resistance. Systemic insulin resistance plays a key role in prediabetes, as it leads to decreased ability of the body to respond to insulin, resulting in an imbalance in glucose homeostasis which, in turn, leads to insulin resistance. The decreased ability of muscle cells to uptake and process glucose reduces the storage capacity for both glucose and triglycerides, resulting in abnormally elevated levels of free glucose and triglycerides in the blood, ultimately increasing the risk of developing diabetes[17,18].

Most prediabetic states progress to diabetes mellitus, accompanied by complications including microvascular complications, retinopathy, and cardiovascular disease. Insulin resistance affects normal oxidative stress in nerves, leading to mitochondrial dysfunction, which causes retinopathy and drives neurological and vascular pathology. The incidence rate of retinopathy is approximately 8.2%-20.9% in prediabetic patients[19-22], while the risk of stroke increases by 0.74% compared to that in patients without diabetes[23]. Additionally, the prevalence of metabolic syndrome is approximately 37.6% higher than that of normoglycemic patients, and the vascular risk ratio score is increased by 0.43[24,25]. Prediabetes is characterized by hyperglycemia and insulin resistance, partly due to the disruption of glucose homeostasis, primarily caused by the compromised function of pancreatic islet β-cells. Adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) is an insulin sensitizer that exists in the form of a heterotrimeric complex with major subunits comprising AMPKα, AMPKβ, and AMPKγ. As blood glucose levels transition from fasting to postprandial levels, the decline in

Table 1 American Diabetes Association diagnostic criteria

Indicator	Numerical range	Ref.
FBG	100-125 mg/dL (5.6-6.9 mmol/L)	[8-11]
IGT	140-199 mg/dL (7.8-11.0 mmol/L)	
A1C	5.7%-6.4% (39-47 mmol/mol/L)	

FBG: Fasting blood glucose; IGT: Impaired glucose tolerance value; A1C: Glycated hemoglobin.

Table 2 Prevalence of prediabetes in China (2008–2018)

Yr	Prevalence in males	Prevalence in female	Total prevalence	Ref.
2008	16.1%	14.9%	15.5%	Wang *et al*[14]
2013	36.4%	35%	35.7%	Wang *et al*[14]
2015-2017	–	–	35.2%	Wang *et al*[15]
2018	–	–	38.1%	Li *et al*[16]

phosphorylated AMPK levels within islets triggers the activation of AMPK phosphorylation, enhancing glucose-stimulated insulin secretion (GSIS). This promoted glucose uptake in muscle tissues while reducing glucose production in the liver to maintain constant blood glucose levels. The activity of AMPK activity is lowest when ATP occupies the subunit site of AMPKγ under high-energy conditions. Liver kinase B1 (LKB1) is required to regulate AMPK activity through AMP/ADP or AMPK phosphatase inhibition. LKB1 primarily phosphorylates AMPKα by binding to Thr172, while LKB1 deficiency in β-cells inhibits the phosphorylation of Thr172 and AMPK target proteins. In contrast, the variable binding of the subunit to AMPKγ, phosphorylation of the downstream kinase Thr172, and impaired downstream dephosphorylation determine the degree of AMPK activation. This kinase in pancreatic β-cells may be protein phosphatase 1, which prevents the sustained activation of AMPK in the presence of high glucose, leading to GSIS failure and insulin resistance[26]. In damaged pancreatic β-cells, a sustained high-glucose environment results in sustained AMPK phosphorylation in the pancreatic β-cells, inhibiting GSIS and promoting insulin resistance.

Apart from hyperglycemia and insulin resistance, prediabetes also presents elevated endoplasmic reticulum (ER) stress levels and abnormal apoptosis of pancreatic islet β-cells. ER stress induces senescence of pancreatic β-cells due to the over-activation of the mammalian target of rapamycin (mTOR), a serine-threonine kinase, encompassing mTOR1 and mTOR2. mTOR1 is responsible for protein synthesis and ribosome genesis, while mTOR2 activates AKT-serine 473. Protein synthesis occurs in the ER.

The unfolded protein response (UPR) is activated when misfolded proteins accumulate in the ER. Over-activation of mTOR1 promotes excessive protein synthesis, increasing the likelihood of misfolded protein synthesis. This, in turn, sustains UPR activation, impairs cellular autophagy mechanism, and leads to pancreatic β-cell death. In patients with prediabetes, prolonged over-activation of the mTOR complex 1 signaling pathway in the β islets results in increased pancreatic β-cell numbers and inhibition of the β-cell autophagy protection mechanism, increasing the likelihood of apoptosis[27].

Insulin resistance and pancreatic islet β-cell apoptosis due to insufficient insulin secretion impedes the normal glucose-lowering effect. Reduced insulin target cell receptor sensitivity leads to diminished insulin signaling, thereby decreasing glucose uptake and increasing extracellular free glucose. Furthermore, the body's negative feedback leads to more insulin release, causing hyperinsulinemia and creating a vicious cycle. The resulting insulin resistance promotes the development of prediabetes. Additionally, excessive free extracellular glucose promotes glucose uptake by the cells, leading to an imbalance in blood glucose homeostasis[27].

The primary preventive measures for prediabetes include lifestyle interventions and pharmacotherapy (Table 3). These interventions primarily aim to reduce glycemic weight, improve insulin resistance, reduce pancreatic β-cell apoptosis, and reduce oxidative stress, thereby reducing islet resistance. Additionally, lifestyle interventions are intended to assist patients with prediabetes in improving unhealthy lifestyles and dietary habits, among others, while naturally reversing the imbalance in blood glucose homeostasis. An advantage of lifestyle interventions is their rapid effectiveness; however, lifestyle regulation is time-consuming[28-30]. Dietary and lifestyle changes can considerably improve the weight and blood glucose levels of individuals who have followed high-fat and-calorie diets over a long time. However, sustained improvement in blood glucose with weight loss may be minimal[30]. Additionally, maintaining a healthy diet over the long term may be challenging for individuals in the contemporary context. Pharmacological management is another form of prediabetes intervention that is remarkably more effective in controlling weight and blood glucose than dietary control. It is also adaptable to modern, high-stress, fast-paced lifestyles[31-36]. The main available drugs include metformin, GLP-1 receptor agonists, SGLT2 inhibitors, vitamin D supplements, and Chinese herbal medicine, among others.

Table 3 Main intervention modalities for prediabetes

Intervention method	Cycle time, follow-up time	Effect	Ref.
Lifestyle intervention	4 months, 1 yr follow up	Blood glucose and lipids can be effectively controlled	Gokulakrishnan *et al*[28]
Lifestyle intervention	1 yr	Effective reduction of disease risk in patients with prediabetes	Hu *et al*[29]
Lifestyle intervention	1 yr	Weight loss 34.1% higher than in the diatomic group	Apolzan *et al*[30]
Metformin	10 yr	Enhanced glycemic control to improve health outcomes	Jonas *et al*[31]
Metformin	1 yr	More effective in body weight reduction. Better results than life interventions	O'Brien *et al*[32]
Metformin	5 yr	Weight loss 2.5% higher than life intervention group	Apolzan *et al*[30]
Metformin	15 yr	Compared with the placebo group, 17% lower incidence of diabetes	Diabetes Prevention Program Research Group[33]
GLP-1 receptor agonist	3 yr	Significant weight loss and improved blood sugar	le Roux *et al*[34]
GLP-1 receptor agonist	17 months	Significant weight loss	Wilding *et al*[35]
GLP-1 receptor agonist	14 wk	Significant reduction of body weight and improved relevant glucose tolerance indicators	Kim *et al*[36]

GLP-1: Glucagon-like peptide-1.

DRUGS FOR PREDIABETES TREATMENT

Metformin

Metformin is a primary hypoglycemic agent that can lower glucose levels by impeding glucose production and enhancing its uptake and utilization[37-39]. Metformin stimulates AMPK, considerably ameliorating abnormalities in glycolipid metabolism[40]. Metformin can promote AMPK phosphorylation, reduce oxidative stress in skeletal muscle, and reverse glucose intolerance, leading to a hypoglycemic effect on the body[41]. Ma *et al*[42] explored the relationship between metformin, presenilin enhancer 2 (*PEN2*), and AMPK by knocking down the *PEN2* gene or reintroducing the *PEN1* mutant gene into *Cryptobacterium*. They reported that metformin can bind *PEN2*, activate ATP6AP1 and AMPK, and initiate glucose metabolism-related signaling pathways, exerting its hypoglycemic effect[42].

AMPK acts as a cellular energy sensor[43,44] and is closely related to the body's activity level. ATP decreases with strenuous exercise, and the ATP/ADP and ATP/AMP ratios subsequently decrease. The concomitant activation of the closely related AMPK positively regulates pathways that replenish the cellular ATP supply, including increasing glucose uptake, activating cellular autophagy, and promoting fatty acid oxidation, negatively regulating biosynthetic processes that consume ATP, including gluconeogenesis[45,46], cholesterol synthesis, protein synthesis, and fatty acid synthesis (Figure 1)[47].

Indeed, metformin effectively reduces the risk of developing diabetes during the prediabetic stage[2,48]. The American Diabetes Association states that metformin is the most effective drug for diabetes prevention and recommends its use for prediabetes intervention[2]. Long-term metformin administration results in marked weight loss in a few patients, with minimal gastrointestinal upset. Therefore, it is considered safe, effective, and well-tolerated for the treatment of patients with prediabetes and abnormally elevated fasting glucose and IGT levels[49].

Metformin is clinically prescribed at a starting dose of 500 mg, which can be increased to 1000 mg twice daily. The dosage varies according to the individualized requirements of the patient. Reported doses used during prediabetic interventions are listed in Table 4. In previous safety trials, patients exhibited symptoms of anemia after long-term use of metformin due to the diminished concentrations of vitamin B12[50]. Therefore, the Diabetes Prevention Program recommends that long-term metformin users should be tested for vitamin B12 levels, with B12 supplementation.

Metformin use has certain shortcomings, including gastrointestinal symptoms, such as abdominal pain and diarrhea, which occur in 30% of users. The incidence of such symptoms increases with the duration of use[49]. Metformin use may also cause lactic acidosis or even death in patients with severe renal impairment (estimated glomerular filtration rate < 30 mL/min/1.73 m). Additionally, metformin is a biologically active molecule with a low environmental decomposition capacity and may cause aquatic environmental contamination[51].

GLP-1 receptor agonists

GLP-1, a large peptide hormone comprising 30 or 31 amino acids, is primarily secreted by distal enteroendocrine L cells, pancreatic α-cells, and the central nervous system. GLP-1 participates in regulating glucose homeostasis by acting on the

Table 4 Dosage schedule for metformin for treating patients with prediabetes

Trial population	Prescribed dosage	Associated or not	Treatment cycle	Reversal rate	Ref.
Adolescents	1000 mg/d	Rosiglitazone (2 mg)	3.9 yr	80% improvement in glucose tolerance	Zinman *et al*[137]
Adolescents	1000 mg/d	No	6 months	45% increase in insulin sensitivity	Srinivasan *et al*[138]
Adults	850 mg/d	No	1 yr	7% reduction in the incidence of diabetes	Andreadis *et al*[139]
Adults	2000 mg/d	No	1 yr	Increased insulin sensitivity ($P < 0.01$)	Malin *et al*[134]
Adults	1500-2000 mg/d	Exenatide (10-20 μg/d)	1 yr	64% improvement in prediabetes remission rates	Tao *et al*[140]

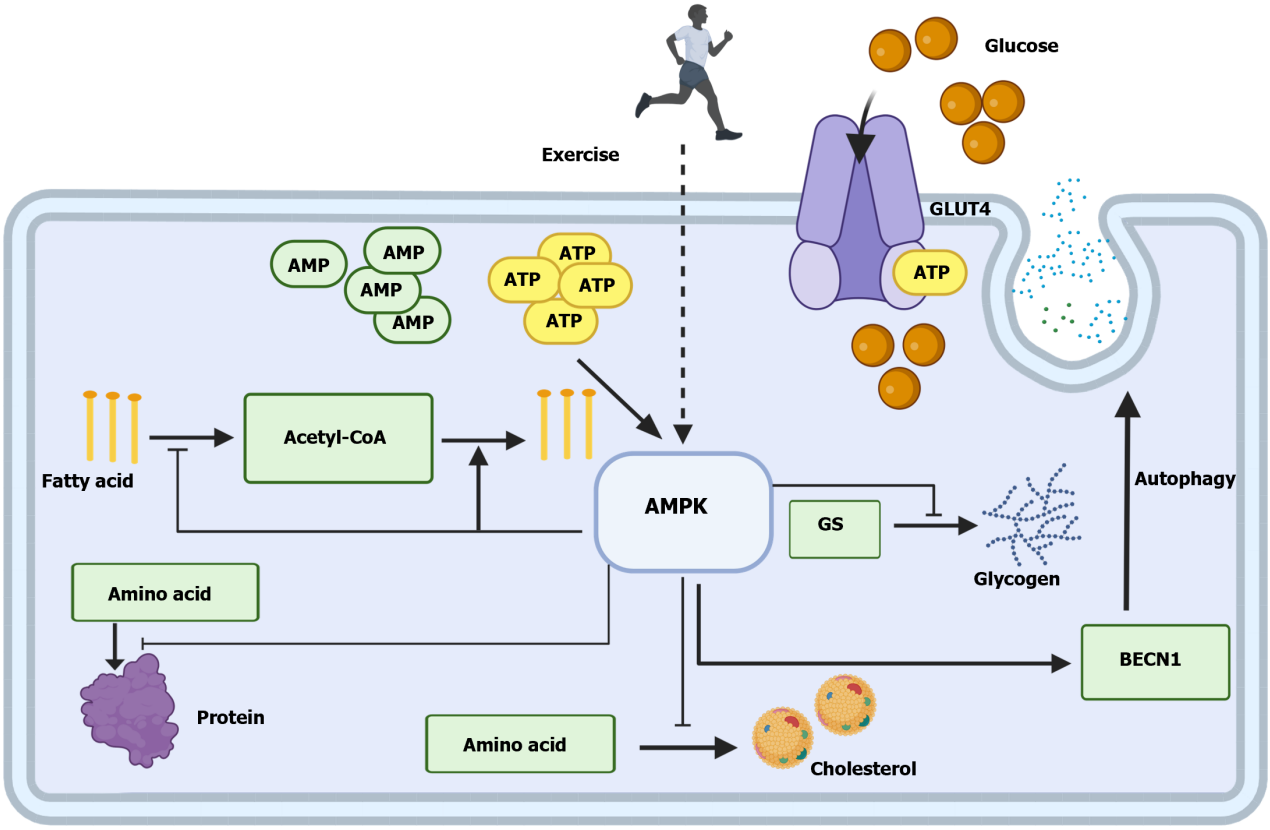

Figure 1 Adenosine 5'-monophosphate-activated protein kinase as an important regulatory center of cellular metabolism. AMP: Adenosine 5'-monophosphate; GLUT4: Glucose transporter type 4; GS: Glycogen synthase; BECN1: Beclin 1; AMPK: AMP-activated protein kinase.

GLP-1 receptor (GLP-1R). GLP-1R agonists (GLP-1RAS) approved for marketing in China primarily include the six types listed in Table 5.

GLP-1 is a type of entero-insulin, a hormone-stimulated and secreted by intestinal food and endothelial cells, respectively, that acts *via* GLP-1R on pancreatic β-cells to generate more intracellular cyclic AMP and ATP, thereby promoting insulin release from pancreaticβ-cells. Additionally, GLP-1 can inhibit abnormal secretion of glucagon from pancreatic α-cells[52]. It regulates glucose abnormalities by lowering HbA1c concentration, promoting insulin secretion from pancreatic β-cells, reducing body weight, contributing to postprandial glucose regulation[53], and reducing glucagon secretion from pancreatic α-cells in a glucose concentration-dependent manner. This inhibitory function is achieved *via* the paracrine effect of the islets[54]. However, GLP-1 glucose regulation is limited due to its short half-life in plasma. Hence, GLP-1RAS was developed to achieve longer-lasting glucose regulation by extending the half-life.

GLP-1RAS promotes the uptake and utilization of glucose *via* several mechanisms[55]. GLP-1RAS can activate pancreatic β-cell GLP-1R by increasing the affinity of GLP-1 to GLP-1R or by binding directly to GLP-1R, promoting insulin secretion by facilitating the conversion of glucose to ATP, enhancing calcium ions inflow and inhibiting K+ outflow from cells (Figure 2)[56]. GLP-1RAS can inhibit glucagon secretion while promoting glucose-dependent insulin secretion owing to its high affinity and similarity to the natural GLP-1RAS and GLP-1, respectively, counteracting the increase in blood glucose caused by diet. The effect of maintaining blood glucose levels in a normal state is known as the entero-insulin effect[57].

Table 5 Types of Glucagon-like peptide-1 receptor agonists currently approved for marketing in China and their recommended clinical dosage

Name	Molecular formula	Number of amino acids	Recommended initial dosage	Recommended dosage for prediabetes	Ref.
Exenatide	$C_{149}H_{234}N_{40}O_{47}S$	39	10 μg/day	10-20 μg/day	Tavlo *et al* [51]
Liraglutide	$C_{172}H_{265}N_{43}O_{51}$	9	0.6-1.2 mg/day	3 mg/day	le Roux *et al* [34]
Dulaglutide	$C_{40}H_{50}N_8O_5$	8	0.75 mg/week	–	–
Lixisenatide	$C_{215}H_{347}N_{61}O_{65}S$	44	10 μg/day	–	–
Polyethylene glycol loxenatide	$C_{210}H_{325}N_{55}O_{69}S(C_2H_4O)_{2n}$	38	0.1 mg/week	–	–
Benarutide	$C_{149}H_{225}N_{39}O_{46}$	29	0.3 mg/day	–	–

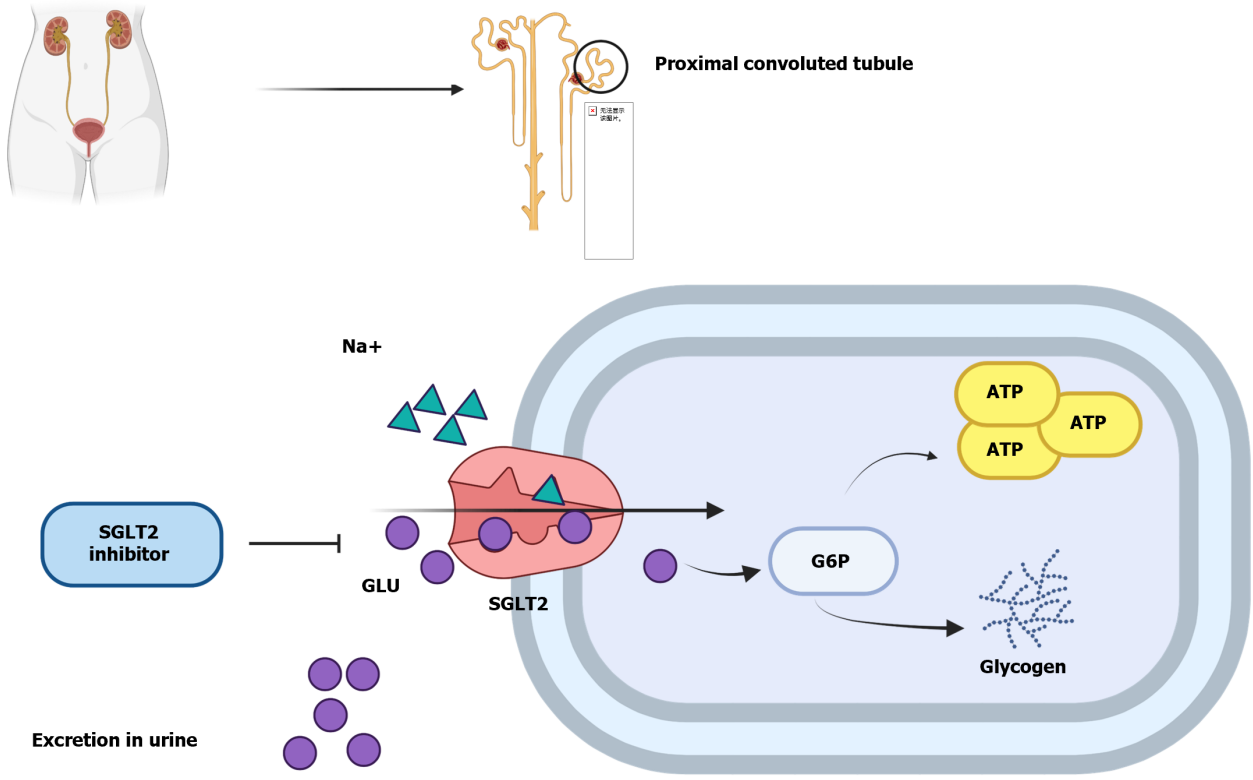

Figure 2 Glucagon-like peptide receptor agonist promotes insulin secretion. G6P: Glucose-6-phosphate; GLP-1: Glucagon-like peptide-1; GLU: Glucose; SGLT2: Sodium–glucose cotransporter 2.

Glucose metabolism is significantly improved after 68 wk of treatment with semaglutide[58]. Oral semaglutide therapy causes HbA1c levels and weight reduction[59]. Meanwhile, tirzepatide upregulates insulin sensitivity in the body and restores islet β-cell function[60]; tirzepatide and semaglutide reduce HbA1c levels[61]. Thus, GLP-1RAS notably aids the restoration of glucose homeostasis, improves islet function, enhances insulin sensitivity, and controls body weight.

Currently, the main adverse reactions associated with GLP-1RAS include nausea, vomiting, and gastrointestinal discomfort[62]. GLP-1RAS is a biomolecular formulation that can only be administered *via* dermal injection. Therefore, it lacks the portability and comfort of small-molecule drugs that can be orally administered.

SGLT2 inhibitors

SGLT 1 and SGLT2 play a prominent role in the reabsorption of filtered glucose by the glomerulus. SGLT2 inhibitors reduce SGLT2 activity and the efficiency of glucose uptake in the proximal tubules of the kidney, which increases the urinary glucose concentration and reduces blood glucose. The main SGLT2 inhibitors currently on the market are listed in Table 6[63-65].

Table 6 Types of Sodium–glucose cotransporter 2 inhibitors and their recommended clinical dosage

Name	Molecular formula	Recommended initial dosage	In China, Listed or not	Recommended dosage for prediabetes	Ref.
Canagliflozin	$C_{24}H_{25}FO_5S$	100 mg/day	No	–	–
Dapagliflozin	$C_{21}H_{25}ClO_6 \cdot C_3H_8O_2$	5 mg/day	Listed	10 mg/day	Lundkvist *et al*[63]
Empagliflozin	$C_{23}H_{27}ClO_7$	10 mg/day	No	10 mg/day	Lee *et al*[64]
Ipragliflozin	$C_{21}H_{21}FO_5S$	50 mg/day	No	–	–
Luseogliflozin	$C_{23}H_{30}O_6S$	2.5 mg/day	No	–	–
Tofogliflozin	$C_{22}H_{26}O_6$	5 mg/day	No	40 mg/day	Pafili *et al*[65]

SGLT2 is an important member of the cotransport protein family. SGLT2 is mainly expressed in the proximal renal tubule, where it facilitates the reabsorption of glucose in the primary filtrate and converts it into ATP or glycogen (Figure 3)[66]. Under normal circumstances, the amount of glycosuria produced by the body after consuming a large quantity of carbohydrates is extremely small, mainly attributed to the filtering and reabsorption ability of SGLT2. SGLT2 inhibitors are a class of hypoglycemic drugs that inhibit the activity of sodium–glucose transport proteins on the luminal surface of the proximal tubule of the kidney, preventing glucose and Na+ from normally entering the cells in the proximal tubule. In addition to lowering blood glucose and body weight, SGLT2 inhibitors improve insulin sensitivity and enhance pancreatic β-cell function, among other effects[39,67-69].

Dapagliflozin and empagliflozin reduce HbA1c by an average of 0.66%[70]. SGLT2 inhibitors delayed the development of diabetes in four randomized trials involving 5655 patients with prediabetes[71]. Moreover, dapagliflozin administration to obese and overweight individuals resulted in weight loss and marked reductions in blood lipids and glucose, including associated OGTTs[72]. These findings show that SGLT2 inhibitors are highly effective in preventing diabetes.

However, the increased glycosuria level caused by SGLT2 inhibitors increases the risk of fungal infections. In eight clinical trials, 3.5% of SGLT2 inhibitor users experienced ketoacidosis. SGLT2 inhibitors may also accelerate the loss of minerals from bone, thereby increasing the risk of fracture. Additionally, SGLT2 inhibitors facilitate Na^+ excretion and may cause adverse effects, such as acute kidney injury and renal function impairment[73,74].

Characteristics of metformin, GLP-1 receptor agonists, and sodium-glucose cotransporter 2 inhibitors use

Prediabetes is treated with medications similar to those used for treating diabetes. Table 7 summarizes the dosages, main results, and related conclusions of the use of metformin, GLP-1 agonists, and SGLT2 inhibitors in individuals with prediabetes, non-diabetic individuals, and individuals with obesity.

Vitamin D

Vitamin D plays an important role in maintaining Ca^{2+} and phosphorus homeostasis, enhancing bone strength[75], increasing bone growth[76], reducing body weight[77], participating in cell differentiation[78], supporting immune function[79] (Figure 4), and delaying the progression of diabetes by lowering blood glucose and maintaining glucose metabolism homeostasis[80,81]. Randomized double-blind and placebo human trials have found that increasing and maintaining serum vitamin D levels reduce the risk of diabetes[82,83].

Recent research indicates that vitamin D reduces the risk of diabetes and its related conditions *via* various mechanisms [84,85]. First, vitamin D promotes insulin synthesis and secretion by improving the function of pancreatic β-cells[86]. Second, vitamin D reduces insulin resistance and improves sensitivity by modulating insulin's target sites (liver, muscle, and adipose tissue)[87].

Research indicates a marked reduction in the insulin resistance index (HOMA-IR) in a vitamin intervention group compared with a placebo group[88]. Additionally, a considerable improvement was found in the glycemic index with dosages of vitamin D > 2000 IU/d.

A study of baseline serum vitamin D concentrations in more than 6000 patients with abnormal blood glucose levels found that individuals with high levels of serum vitamin D have a considerably reduced prevalence of elevated blood glucose and associated complications compared with those with serum vitamin < 25 nmol/L[89]. Another study in 2423 individuals with prediabetes identified the lowest risk of diabetes in individuals with serum vitamin D levels of ≥ 125 mmol/L; serum vitamin D levels of 100-124 mmol/L reduced the risk of developing diabetes in some individuals[90]. Furthermore, 43 randomized controlled trials have reported that high doses of vitamin D (≥ 1000 IU/d) markedly reduced the risk of developing diabetes in 55936 individuals with prediabetes[91]. Four trials, including 896 participants, have found that vitamin D supplementation effectively reduces the risk of prediabetes progressing to diabetes[83,92].

Vitamin D use for therapeutic interventions may cause hypercalcemia[93]. Vitamin D is present in the body mainly as vitamins D2 or D3, which are not biologically active. The inactive forms are catabolized and metabolized in the liver to 25-hydroxyvitamin D (ossified diol) and the kidney to 1,25-dihydroxy vitamin D (ossified triol), with both metabolites being biologically active[94]. Osteotriol is the main metabolite of vitamin D in the body and mediates Ca^{2+} and phosphorus uptake. Excessively elevated osteotriol levels can lead to hypercalcemia and hyperphosphatemia, which increases the risk of vascular calcification. Therefore, phosphate levels should be strictly monitored with vitamin D intervention.

Table 7 Types of Sodium–glucose cotransporter 2 inhibitors and clinical research results

Name	Ref.	Participants	Grouping and dosage	Result	Conclusion
Metformin	O'Brien *et al*[32]	92	Metformin group (850 mg/daily), standard diet group	Compared with the standard diet group, the metformin group lost an average of 1.1% body weight, and a normal blood glucose ratio of 28.7% was restored	Reduces weight and restores normal blood glucose levels in prediabetics
Metformin	Tavlo *et al*[51]	183	1500-2000 mg/d over 12 wk	The impaired glucose tolerance remission rate was 32%	Improves postprandial insulin secretion
Metformin + exenatide	Tavlo *et al*[51]	183	Metformin: 1500-2000 mg/d; exenatide: 10-20 μg/d over 12 wk	The impaired glucose tolerance remission rate was 64%	Combined administration of drugs is more effective in alleviating glucose tolerance compared with monotherapy
Exenatide	Tavlo *et al*[51]	183	10-20 μg/d over 12 wk	Impaired glucose tolerance remission rate of 56%	Improves postprandial insulin secretion
Liraglutide	le Roux *et al*[34]	749	Placebo group (n = 749), liraglutide group (n = 1505, 3 mg/d) over 160 wk	4.2% weight loss and 2.7 times longer onset of diabetes in the liraglutide group than the placebo group	3 mg liraglutide reduces weight gain and diabetes risk
Liraglutide	Pi-Sunyer *et al*[141]	3731	Placebo (n = 1244), liraglutide (n = 2487, 3 mg/d) over 56 wk	Body weight in the liraglutide group decreased by 36.1%; glycated hemoglobin, fasting blood glucose, and fasting insulin were reduced; and the prevalence of diabetes was reduced, compared with the placebo group	3 mg liraglutide may reduce the incidence of urine disease
Dapagliflozin	Veelen *et al*[142]	30	Dapagliflozin (2 mg/d) *vs* placebo, over 10 wk	The plasma glucose level was reduced in the dapagliflozin group compared with the placebo group, and no extensive changes were observed in the glycogen and lipid content of the liver	Dapagliflozin improves fat oxidation and exhibits a marked hypoglycemic effect

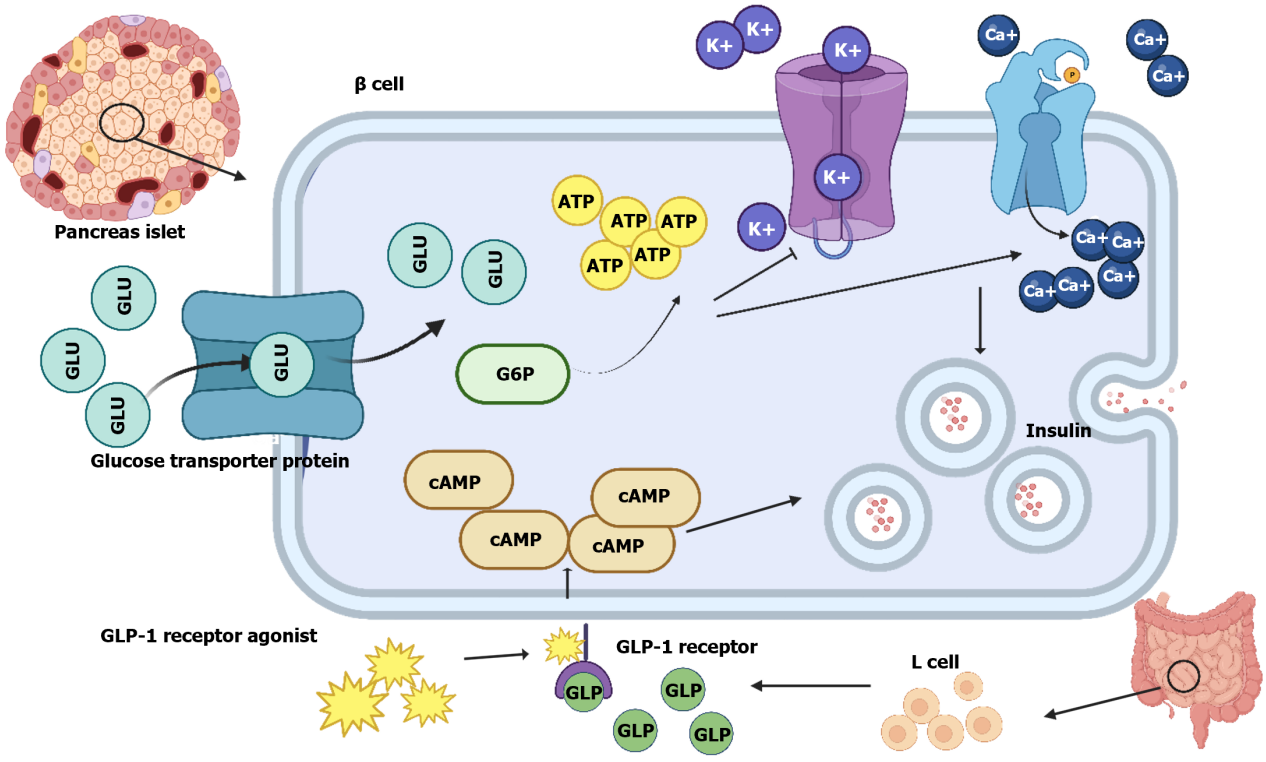

Figure 3 Sodium–glucose cotransporter 2 inhibitors block the glucose reabsorption process. G6P: Glucose-6-phosphate; GLP-1: Glucagon-like peptide-1; GLU: Glucose; SGLT2: Sodium–glucose cotransporter 2.

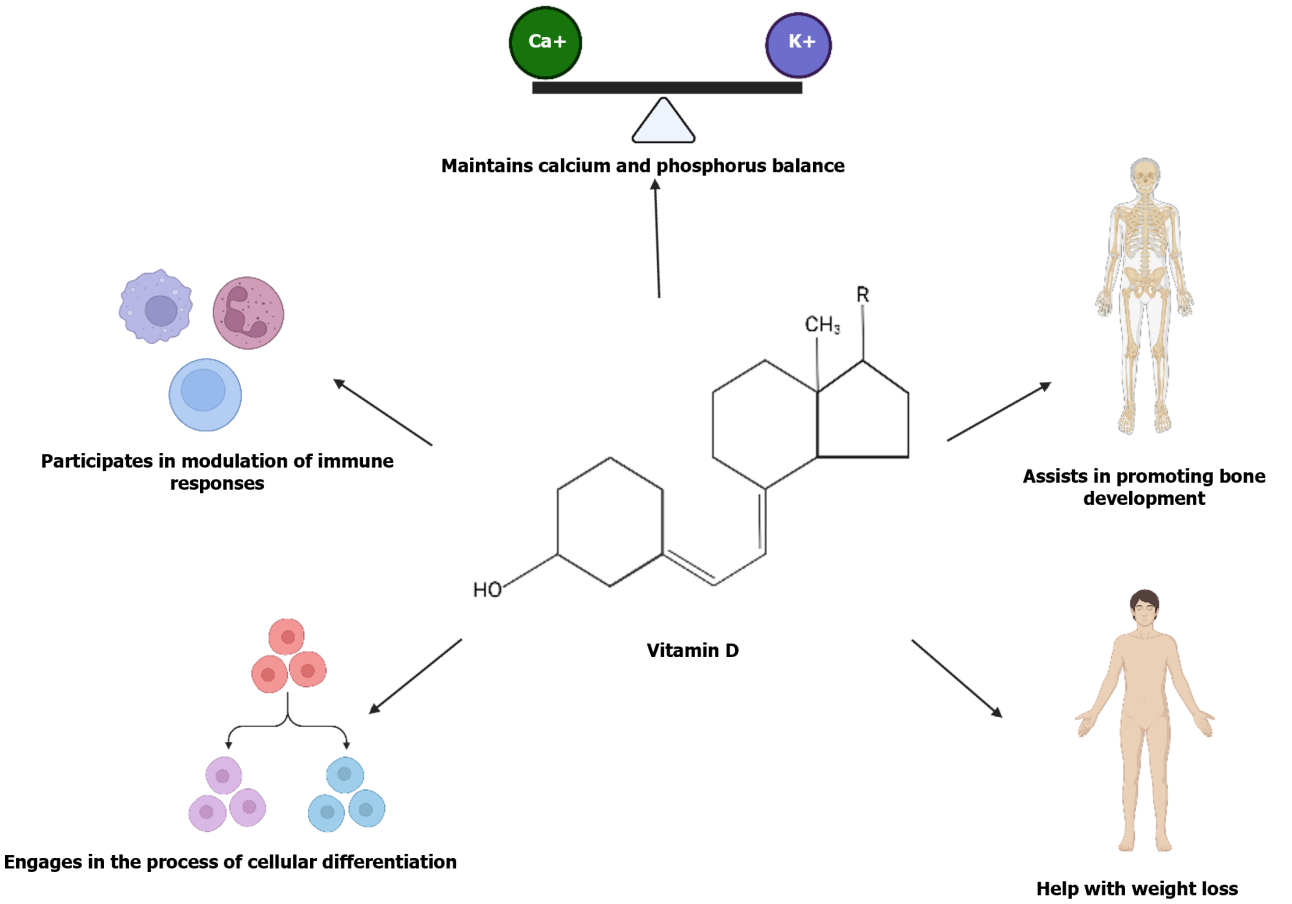

Figure 4 Vitamin D contributes to the maintenance of normal bodily functions.

Patients may also include vitamin D-enriched foods in their daily dietary regimen, such as those listed in Table 8[95].

Chinese herbal medicine

The use of herbal medicine for treating diabetes and prediabetes dates back to the Qin Dynasty (approximately 221 BC). Currently, herbal medicines are considered useful for preventing and treating diabetes and its complications[96]. The herbs used for diabetes and prediabetes generally have multiple effects, including counteracting hypoglycemia, reducing insulin resistance, reducing oxidative stress, lowering lipids, and regulating intestinal flora. Numerous Chinese herbal medicines are currently used for treating prediabetes, including Huanglian, Qingqianliu, Mulberry [*Morus alba* (*M. alba*)] leaf, Astragalus, Guajia, Lady's mantle, Dendrobium, Dry lotus, Ginseng, Wolfberry, Pentaphyllum, and Sanguisorba [97]. The functions and mechanisms of action of the herbs, including berberine, safranin, cyanotis, *M. alba* leaf, and Astragalus, in alleviating diabetes and prediabetic symptoms are briefly discussed here.

Berberine, a main active ingredient in Huanglian used in treating diabetes and prediabetes, plays crucial roles in treating hypoglycemia and other aspects. It positively affects mucin increase and promotes the improvement of intestinal mucosal morphology. Additionally, berberine can down-regulate the expression of Toll-like receptor 4, nuclear factor kappa B (NF-κB), and tumor necrosis factor-α, alleviating the chronic inflammation caused by diabetes. Furthermore, it counteracts the reduction of intestinal microbial diversity caused by IGT; reduces FBG and HOMA-IR; improves liver and kidney function; reduces cholesterol, blood lipid, and high-density fatty acid levels; and increases the number of cupped cells and villi length in IGT rats[98,99]. Berberine induces accelerated closure of KCNH6 K+ channels, decreases KCNH6 currents, prolongs glucose-dependent cell membrane depolarization, and promotes insulin secretion[100]. However, it exhibits an IC_{50} of 713.57 mg/kg in acute toxicity tests in rats[101]. Therefore, the use of berberine as an alkaloidal constituent of *Coptis chinensis* should be approached with due consideration of its toxicity.

Phellodendrin (PAL) is an active constituent of *Phellodendron* that improves blood glucose and insulin resistance levels in rats with IGT. Furthermore, PAL ameliorates the defective insulin secretion in insulinoma cells induced by chondroitin (PA) *via* the c-Jun N-terminal kinase signaling pathway. It also extensively inhibits PA-induced cell-induced β-cell apoptosis[102]. However, PAL elicits toxic effects with an IC_{50} of 1533.68 mg/kg in acute toxicity tests in rats[101]. Accordingly, attention to dosage is required in PAL use for prediabetes prophylaxis[103].

Cycads have various therapeutic properties, including hypoglycemic, hypolipidemic, hypotensive, anticancer, anti-fatigue, and antioxidant effects[104,105]. *Cyanus* was used in ancient times to treat diabetes[106-108]. Cyanidin improves insulin secretion by reducing apoptosis of pancreatic β-cells, reducing excessive oxidative stress in the pancreas, and maintaining the balance of glucose and lipid metabolism in the liver, thereby regulating blood glucose and lipid regulatory homeostasis[109]. *Cryptococcus* can also relieve hyperglycemic symptoms by modulating the intestinal

Table 8 Selected vitamin D-rich foods and their vitamin D content

Food	Vitamin D content
Fresh shiitake mushrooms (0.0992 kg)	600-1000 IU D2
Sun-dried shiitake mushrooms (0.0992 kg)	600-1000 IU D2
Egg yolk	20 IU D3, 0.2-0.8 μg 25-(OH)D
Cod liver oil (0.006 kg)	400-1000 IU D3
Beef liver (0.4536 kg)	0-2500 IU D3, 0.3-3.5 μg 25-(OH)D
Beef muscle (0.4536 kg)	0-180 IU D3, 0.1-2.6 μg 25-(OH)D
Pork muscle (0.4536 kg)	10-250 IU D3, 0-31.4 μg 25-(OH)D

IU: International Unit; D2: vitamin D2; D3: vitamin D3.

microflora[110,111]. *Cyclocarya paliurus* is a traditional medicinal plant with various active effects; however, its safety issues should not be overlooked. Rats have shown good tolerance in acute toxicity studies; however, adverse changes in hematology, serum chemistry, urinalysis parameters, organ weights, and histopathology occur. Currently, *C. paliurus* is regarded as safe for use in the treatment of prediabetes[112].

M. alba is an Asian medicinal plant with roots, fruits, and seeds used to lower glucose levels, reduce liver damage, and improve oxidative stress[113,114]. Flavonoids, polysaccharides, and alkaloids are key active components in *M. alba* leaves that alleviate symptoms of hyperglycemia. *M. alba* leaf extract intervention in mice with IGT reduces insulin resistance and IGT while improving glucose uptake in a hepatocyte islet resistance model[115]. *M. alba* extract considerably improves glucose lipid levels, islet function, and insulin resistance index. It also substantially inhibits PA-induced apoptosis and markedly activates the AMPK/mTOR signaling pathway, inducing islet cell autophagy and improving the functional utilization of islet cells[115,116]. The aqueous extract of *M. alba* downregulates the expression levels of relevant inflammatory factors, ameliorating chronic inflammation. Additionally, *M. alba* eliminates oxidative stress caused by IGT by modulating the advanced glycation end-products (AGEs)/receptor of AGEs/NADPH oxidase 4/NF-κB signaling pathway[117]. Importantly, ensuring the safety of *M. alba* leaves is crucial due to the abundance of biologically active phytochemicals and their many beneficial components. Acute toxicity, subacute toxicity, and genotoxicity studies in rats have shown no mortality or abnormal behavioral changes; no parameter changes in blood, biochemistry, or histopathology; and no mutagenic activity in the Ames assay. These findings weaken the claim that the aqueous extract of *M. alba* leaves may induce chromosomal aberrations or sperm abnormalities[118]. Therefore, the medicinal use of the aqueous extract of *M. alba* leaves is considered safe.

Astragalus membranaceus (A. membranaceus) has a long history of medical applications in China, including tonifying qi, lowering lipid and blood pressure levels, nurturing the heart, and regulating blood glucose[119,120]. Moreover, flavonoids of *A. membranaceus* regulate intestinal microflora, reduce FBG, and improve brain damage caused by IGT[121]. Water-soluble *A. membranaceus* polysaccharides considerably reduce blood glucose levels and the insulin resistance index. They enhance glucose intolerance; improve insulin resistance in mice; and reduce oxidative stress, inflammation, and liver injury, while increasing the concentration of short-chain fatty acids in the intestinal flora. Notably, they augment the levels of *Allobaculum, Lactobacillus, Akkermansia*, *Faecia, Akkermansia*, and *Faecalibaculum* in the intestinal flora of mice with IGT[122,123]. These functions have positive implications for alleviating symptoms associated with diabetes[124]. Unfortunately, studies on the toxicity of *A. membranaceus* are limited, and toxicology tests are required before considering it as an intervention for prediabetes.

BEHAVIORAL INTERVENTION

In addressing prediabetes, obesity is a key factor. To delay diabetes onset, increasing exercise intensity and duration is crucial. For prediabetic patients, a weekly increase of 150 min in exercise or 30 min daily can significantly lower the fasting glycemic index[125]. Six months of high-intensity exercise effectively improves oral glucose tolerance[126], and 20 wk of sustained exercise normalizes blood glucose levels[127]. A Meta-analysis confirms that both aerobic and resistance training, individually or combined, benefit insulin resistance and glycemic control in prediabetic patients[128].

Enhancing behavioral interventions requires a comprehensive, adaptable strategy that accounts for patient preferences, risks, and comorbidities, ensuring long-term adherence. This strategy should include building supportive relationships that encourage healthy behaviors, timely plan adjustments based on patient progress, and incorporating incentives for sustained motivation and adherence[129].

DIETARY INTERVENTION

A high-calorie diet contributes to prediabetes development. Epidemiologic evidence supports increasing intake of non-starchy vegetables, fruits, and whole grains[130], while limiting added sugars to effectively reduce glycated HbA1c, fasting glycemic index, serum insulin, insulin resistance, cholesterol levels, body weight, and body mass index[131]. This approach also lowers type 2 diabetes risk[132]. Early time-restricted feeding, involving a 6-h eating window ending by 3 p.m., improves insulin sensitivity, β-cell responsiveness, blood pressure, oxidative stress, and appetite within 5 wk, aiding diabetes prevention[133]. This study aims to concisely highlight the importance of dietary protein and fiber in mitigating prediabetes.

PHARMACOLOGICAL AND LIFESTYLE INTERVENTIONS

Pharmacologic and lifestyle interventions can be combined for the prevention of diabetes. Metformin is the most commonly used drug in combination with lifestyle interventions. The administration of metformin (500-2000 mg/d) combined with exercise has improved insulin sensitivity in prediabetic patients[134]. However, this combination does not offer an advantage[135] and may even diminish the glucose-lowering effects of exercise[136] compared with metformin alone (1000 mg twice daily) and exercise training alone.

DISCUSSION

Diabetes has a high incidence with a low reversal rate[137]. Prediabetes, a common precursor often accompanied by microvascular complications like retinopathy, cardiovascular disease, and other issues, underscores the importance of effective intervention and management to slow or even prevent the development of diabetes.

Unhealthy lifestyles play a pivotal role in prediabetes development. Achieving complete remission of prediabetes necessitates the long-term maintenance of a healthy lifestyle. A combination of a nutritious diet and increased physical activity plays a crucial role in preventing or delaying the onset of diabetes and its complications. While lifestyle interventions are effective in the short term, their long-term efficacy is limited. Therefore, pharmacological interventions become necessary. These interventions can correct glucose homeostasis dysregulation, delay diabetes progression, and control glucose and lipid metabolism disorders. Common pharmacological interventions for prediabetes include metformin, GLP-1RAS, SGLT2 inhibitors, vitamin D, and Chinese herbal medicine[138-142]. These drugs have multifaceted effects, including blood sugar regulation, improved insulin sensitivity, and reduced insulin resistance.

Current treatments for prediabetic patients predominantly consist of lifestyle and pharmacological interventions. However, patient adherence to lifestyle interventions is often challenging to maintain in the long term, and a single lifestyle change is typically insufficient to extensively improve glycemic regulation. In contrast, a single pharmacological intervention can promptly lower blood glucose levels and enhance insulin sensitivity, yet prolonged use may lead to drug resistance over time. Combining lifestyle interventions with appropriate medication, as opposed to monotherapy, can yield a more favorable therapeutic outcome by promoting sustained weight loss, normal blood glucose control, pancreatic islet cell repair, and improved insulin sensitivity. Integrating lifestyle and pharmacological interventions is likely to be more acceptable to prediabetic patients and aligns with current treatment trends. Furthermore, the prevention and management of prediabetes take precedence over treating diabetes. Regular monitoring of daily blood glucose, weight, and medical parameters enables timely diabetes detection and disease progression control. Safe and effective interventions for prediabetes remain a necessity. Future efforts should focus on improving standardized prediabetes diagnosis to facilitate early detection, management, and treatment of prediabetic patients. The integration of pharmacological and lifestyle interventions is poised to become a new direction in prediabetes treatment.

CONCLUSION

This paper provides a comprehensive overview of the mechanisms behind prediabetes development and its associated therapeutic drugs. Prediabetes is significantly influenced by unhealthy lifestyles. To achieve complete remission, it is crucial to maintain a healthy lifestyle, including a balanced diet and regular physical activity. These practices are key in preventing or delaying the onset of diabetes and its complications. While lifestyle changes are effective short-term, their long-term efficacy is limited, making pharmacological treatments essential. Treatments such as metformin, GLP-1RAS, SGLT2 inhibitors, vitamin D, and Chinese herbal medicine play a pivotal role in regulating glucose homeostasis, decelerating diabetes progression, and controlling glucose and lipid metabolism disorders. They also help regulate blood sugar levels, improve insulin sensitivity, and reduce insulin resistance.

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Author contributions: Ping WX conceived, designed, and refined the idea of the article; Hu S contributed ideas; and all authors participated in, read, and approved the final manuscript. Ping WX and Hu S contributed equally to this review as co-first authors. Ping WX and Hu S were designated as co-first authors for two reasons. First, this review was a collaborative effort, and the designation of co-first authors accurately reflects the responsibility for the effort required to complete the paper. It also ensured effective communication and post-submission management, which ultimately improved the quality and reliability of the paper. Secondly, the overall team included authors from different fields with a variety of expertise and skills, and the designation of co-corresponding authors reflected this diversity. This also contributed to the most comprehensive and in-depth exploration of this review, ultimately enriching the reader's understanding by providing different expert perspectives. The selection of these two individuals as co-first authors recognizes and respects their equal contribution and acknowledges the collaborative spirit of this team.

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REVIEW

Epigenetic modifications of placenta in women with gestational diabetes mellitus and their offspring

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Abstract

Gestational diabetes mellitus (GDM) is a pregnancy-related complication characterized by abnormal glucose metabolism in pregnant women and has an important impact on fetal development. As a bridge between the mother and the fetus, the placenta has nutrient transport functions, endocrine functions, *etc.*, and can regulate placental nutrient transport and fetal growth and development according to maternal metabolic status. Only by means of placental transmission can changes in maternal hyperglycemia affect the fetus. There are many reports on the placental pathophysiological changes associated with GDM, the impacts of GDM on the growth and development of offspring, and the prevalence of GDM in offspring after birth. Placental epigenetic changes in GDM are involved in the programming of fetal development and are involved in the pathogenesis of later chronic diseases. This paper summarizes the effects of changes in placental nutrient transport function and hormone secretion levels due to maternal hyperglycemia and hyperinsulinemia on the development of offspring as well as the participation of changes in placental epigenetic modifications due to maternal hyperglycemia in intrauterine fetal programming to promote a comprehensive understanding of the impacts of placental epigenetic modifications on the development of offspring from patients with GDM.

Key Words: Gestational diabetes mellitus; Placental functions; Epigenetics; Offspring development

Core Tip: Gestational diabetes mellitus is a pregnancy-related complication characterized by abnormal glucose metabolism in pregnant women and has an important impact on fetal development. The review aims to investigate the effect of abnormal placental function on offspring development in pregnant women with gestational diabetes from the perspective of epigenetics.

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INTRODUCTION

Gestational diabetes mellitus (GDM) is a common metabolic disorder during pregnancy and refers to an abnormal glucose tolerance that occurs or is first observed during pregnancy. Epidemiological evidence shows that in recent years, the prevalence of GDM has been on the rise worldwide. The international prevalence rate of GDM varies from 6.6% to 45.3%[1], depending on the region, population and diagnostic criteria and the total prevalence rate of GDM in China is 14.8%[2]. Like type 2 diabetes, GDM is characterized by relative insulin deficiency caused by changes in the function and mass of β cells and an increase in insulin resistance[3]. The offspring of patients with GDM are more prone to suffer from congenital developmental abnormalities[4,5] and complications such as macrosomia, hypoglycemia, hyperbilirubinemia, respiratory distress syndrome, and later obesity, metabolic disorders and cardiovascular abnormalities[6,7]. GDM has become a public health issue of global concern.

The placenta plays a crucial regulatory role in maintaining fetal growth and development throughout pregnancy, as it has multiple functions, such as nutrient transport and endocrine functions. Moreover, abnormal placental functions can also induce a variety of fetal diseases and complications, such as fetal overnutrition or growth restriction. Previous studies have shown that metabolic abnormalities in GDM patients could damage the structure, morphology and functions of the placenta and lead to pathological changes, affecting the energy conversion between the mother and the fetus, and affecting fetal development[8]. In recent years, an increasing number of studies have shown that the placenta is associated with diseases such as obesity in offspring[9], cardiovascular diseases[10] and impaired neurodevelopment[11], implying the importance of the placenta during fetal development.

Studies have shown that pregnant women who are exposed to adverse conditions for a long time, such as smoking, alcohol abuse, lack of exercise, sleep deprivation, unhealthy dietary habits, and hormone use, may also experience changes in the epigenetic level of their placenta, leading to abnormal gene expression, which further results in alterations in placental function and metabolism[12] and an increase in the risk of GDM onset. GDM can also lead to epigenetic abnormalities in the placenta, such as changes in DNA methylation and miRNA expression, thereby affecting normal fetal development[13,14].

In this paper, with the placenta regarded as an important target organ through which GDM affects offspring development, the potential impacts of its functional and epigenetic changes on offspring development are reviewed, and the possible underlying mechanism is explored, providing a scientific basis for preventing abnormal development and ensuring the subsequent health of the offspring of patients with GDM.

IMPACTS OF GDM ON PLACENTAL TRANSPORT FUNCTION

The placenta is a transient multifunctional organ responsible for the transport of nutrients from the mother to the fetus. GDM can affect the nutrient transport function of the placenta, increase or decrease the amount of energy delivered to the fetus, and thus affect fetal weight[15] (Table 1).

Impacts on placental glucose transport

Glucose is the main energy source for the fetus and the placenta. The placental glucose transport function is affected by maternal glucose concentration, glucose transporters (GLUTs) and placental glucose metabolism[15] and is also regulated by insulin in early pregnancy[16]. For patients with GDM, the concentration of GLUT and glucose uptake in the basement membrane (BM) of the placenta increase, and the transport of maternal glucose to the fetus increases, leading to macrosomia[17,18]. However, a decrease in or deficiency of placental GLUT leads to abnormal conditions, such as fetal hypoglycemia and weight loss[19], indicating that placental GLUTs are crucial for fetal growth and development.

In the full-term placenta of GDM patients, the expression levels of GLUT-1, GLUT-4, and GLUT-9 increase and are positively correlated with fetal birth weight[17]. For GDM patients treated with insulin, placental glucose uptake and transport increase, and the expression level of GLUT-4 in the placenta is positively correlated with birth weight and subscapular fat thickness[17]. Similar findings were observed in animal models, *e.g.*, in the placenta of mice with GDM induced by a high-fat diet, the AMPK-GLUT-3 axis was impaired, and the expression of GLUT-3 in the placental plasma membrane decreased, resulting in reduced glucose uptake by the placental trophoblast and excessive glucose input into the offspring, which led to the overgrowth of the offspring[20]. These research results indicate that an intrauterine

Table 1 Relationship between placental transport function and fetal weight in gestational diabetes mellitus

Nutrient	Transporter	GDM model		Cell lines	Mechanism of action	Localization	Result	Ref.
		Gestational age	Animal species					
Glucose	GLUT1	37+ wk gestation	-	-	-	P↑; BM↑	FBW (+); AFM (+), SSFM (+)	[17,18]
		Full-term placenta	-	-	p-Akt and Erk↑	P↑	FBW↑	[23]
	GLUT4	37+ wk gestation	-	-	-	P↑	SSFM (+); FBW (+)	[17]
	GLUT9	37+ wk gestation	-	-	-	P↑	FBW (*)	[17]
	GLUT3	-	db/+mice & HFD-induced C57B L/6J mice	-	AMPK↓	PM↓	FBW↑	[20]
Amino acids	System A	-	-	Insulin stimulates PHT cell and PVE	p-Akt and Erk↑	PHT↑	FBW↑	[9]
		-	-	TNF-α stimulation PHT cell	Erk; p38MAPK	PHT↑	FBW↑	[30]
		-	-	IL-6 treat PCT	JAK/STAT	PCT↑	FBW↑	[31]
		-	-	LPS and poly (I:C) treat PCT	TLR3 and TLR4↑	PCT↑	FBW↑	[32]
	SNAT 1	37-41^{+6} wk gestation	-	-	IGF-I and mTOR↑	P↑	FBW (+)	[25]
		-	-	TNF-α stimulation PHT cell	Erk; p38MAPK	PHT↑	FBW↑	[30]
		-	-	LPS and poly (I:C) treat PCT	TLR3 and TLR4↑	PCT↑	FBW↑	[32]
	SNAT2	-	-	TNF-α stimulation PHT cell	Erk; p38MAPK	PHT↑	FBW↑	[30]
		-	-	IL-6 treat PCT	JAK/STAT	PCT↑	FBW↑	[31]
		-	-	LPS and poly (I:C) treat PCT	TLR3 and TLR4↑	PCT↑	FBW↑	[32]
	SNAT3	-	-	IL-6 treat PCT	JAK/STAT	PCT↑	FBW↑	[31]
	System L	-	-	MVM and BMs from GDM	-	MVM↑	FBW↑	[28]
		-	STZ-induced SD rats	-	mTORC1↓	P↓	FBW↓	[46]
Lipids	TG	-	-	Hight glucose and insulin treat PHT	-	PHT↑	FBW↑	[35]
		-	HF/HCD induced C57BL/6J mice	-	CEH↑, TGH↓	P↑	FBW↑	[36]
		37-42 wk gestation	-	Hight glucose treat PE	β-oxidation↓	P↑	FBW↑	[38]
	EL	Full-term placenta	-	-	p-Akt and Erk↑	P↑	FBW↑	[41]
	FAT	-	-	Hight glucose and insulin treat PHT	-	PHT↑	FBW↑	[35]
		Full-term placenta	-	-	p-Akt and Erk↑	P↑	FBW↑, FBW (+)	[41,42]

FABP4 A-FABP L-FABP	Full-term placenta	-	-	p-Akt and Erk↑	PHT↑, P↑	FBW↑	[34,40, 41]
FABP3, FABP4	-	-	Hight glucose and insulin treat PHT	-	PHT↑	FBW↑	[35]
FATP-1	Full-term placenta	-	-	p-Akt and Erk↑	P↑	FBW↑	[41]

P: Placenta; FBW: Fetal baby weight; SSFM: Subscapular fat mass; AFM: Abdominal; PM: Plasma membrane; BM: Basement membrane; PHT: Primary human trophoblast; PVE: Placental villous explants; PE: Placental explants; PTC: Primary trophoblast cells; CEH: Cholesterol ester hydrolase; MVMs: Microvillous plasma membranes; +: Positive correlation; *: Correlation; FATPs: Fatty acids transport proteins.

hyperglycemic environment alters the expression of placental GLUTs and increases glucose transport between mothers and fetuses, thereby increasing offspring weight.

In addition, insulin can regulate glucose metabolism to promote fetal development in early pregnancy. Studies have shown that the phosphorylation of the insulin-like growth factor 1 receptor (IGF-1R) and increased expression of insulin receptor A (IR-A) in the placenta of GDM patients are associated with fetal overgrowth[21]. The ability of IGFBP to bind to the umbilical cord and placental stroma of patients with GDM is reduced, resulting in an increase in free IGF-1[22]; however, an increase in IGF-1 can activate insulin/IGF-1 signaling (Akt and Erk) in the placenta to increase placental GLUT-1 expression and fetal birth weight[23]. GLUT-4 in placental microvillous membranes (MVMs) increases placental glucose uptake under the regulation of insulin during early pregnancy, leading to an increase in glucose transfer to the fetus[16]. These results indicate that maternal hyperinsulinism regulates the activity and expression of placental GLUTs, which may accelerate fetal growth.

Impacts on placental amino acid transport

Placental amino acid transport is mediated by proteins expressed in maternal-circulation-oriented MVM and fetal-circulation-directed BM. The placenta has 15 amino acid transport systems, such as system A, which is responsible for supplying small neutral amino acids (a Na+-dependent transport protein), and system L, which is responsible for supplying essential large neutral amino acids (a broad Na+-independent transporter protein)[24]. The activation of placental IRs by maternal hyperinsulinism leads to the activation of mammalian target of rapamycin (mTOR)[9,25], which is a key regulatory factor for placental amino acid transport[26] and can promote cell proliferation and fetal growth. There are significant differences between the concentrations of maternal amino acids and the concentrations of amino acids in cord blood from GDM patients, even with well-controlled blood glucose[27], indicating that GDM alters placental amino acid transport or metabolism.

In the case of fetal overgrowth, the ability of the placenta to transport amino acids is significantly improved in GDM patients[28]. The signaling activities of IGF-I and mTOR in the placentas of GDM patients with well-controlled blood glucose increased and were positively correlated with birth weight. In particular, the upregulation of the system A amino acid transport protein in the placenta increased the probability of macrosomic babies occurring in women with GDM[25]. Through experiments on primary human trophoblasts (PHTs) and placental villous explants, maternal hyperinsulinism was shown to activate placental IR signaling (Erk and Akt) pathways and improve amino acid transport in system A[9]. In the placenta of GDM patients, the mTOR signaling pathway is activated, pro-oxidant/pro-inflammatory factors increase[29], and the proinflammatory cytokines TNF-α[30] and IL-6[31] can upregulate the amino acid transport of system A in PHT by activating the Erk/p38 MAPK and JAK/STAT signaling pathways, respectively. The activation of the Toll-like receptor 3 (TLR3) or TLR4 signaling pathway could lead to insulin resistance in primary trophoblast cells and significantly increase the expression of system A amino acids (SNAT1 and SNAT2) and the uptake of related amino acids[32]. Other studies have shown that activation of the TLR4 signaling pathway is associated with increased uptake of system A amino acids stimulated by fatty acids (FAs) in PHT[33]. The L-system, another important placental amino acid transport system, is also involved in fetal weight programming. An increase in L-system-mediated leucine uptake in the placental MVM of a GDM patient with a baby large for gestational age (LGA) promoted placental leucine transport and facilitated the acceleration of fetal growth[28]. These results indicate that GDM increases the transport of amino acids in the placental system, leading to increased risks of fetal overgrowth and obesity.

Impacts on placental lipid transport

The essential FAs required for fetal growth mainly rely on maternal supply and placental transport. Placental FA transport relies mainly on the activity of lipid hydrolases in the syncytiotrophoblast MVM and FA uptake by various FA transport proteins [FA transporters (FATs), FA binding proteins (FABPs), and FA transport proteins (FATPs), *etc.*] in the plasma membrane[34-36].

The high expression levels of the placental proteins PI3K p110α, LXRα, FAS, and SCD1, which are related to lipid metabolism and lipoprotein lipase (LPL), in GDM may lead to the accumulation of placental triglycerides (TGs)[37]. Although the uptake and transport of placental FAs are not affected by maternal hyperglycemia, hyperglycemia reduces the β-oxidation of the placenta and thus leads to an increase in the placental TG[38]. Experiments on human placental explants have shown that the activity of carnitine palmitoyltransferase is inhibited by hyperglycemia, such that β-oxidation is reduced and esterification pathways are increased, leading to the accumulation of placental TG[39]. Another

animal experiment showed that maternal mice fed a high-fat/high-cholesterol diet (accompanied or not accompanied by GDM) had dysregulated placental lipid hydrolase activity, increased cholesteryl ester hydrolase activity, and decreased TG hydrolase activity; as such, excessive cholesterol was input into the offspring, resulting in an increase in liver lipids and the accumulation of placental TG, which may cause overgrowth[36]. These findings indicate that the oxidation of FAs is reduced and that the expression of placental proteins and TG hydrolases becomes imbalanced, which causes the deposition of placental TG; moreover, although maternal TG does not pass through the placenta, it can be decomposed by placental LPL, TG hydrolases and other lipases and subsequently infiltrate the placenta. A series of factors are associated with fetal overgrowth.

It has been reported that the expression of FABP4 is increased and that the expression of LPL is decreased in the male placenta of a GDM patient with macrosomia. Additionally, the mRNA expression level of angiopoietin-like protein 3 (ANGPTL3) is increased, and the activity of LPL is inhibited by ANGPTL3, which leads to an increase in the storage of liver adipocytes; moreover, FABP4 increases the FA gradient to promote the delivery of placental lipids to the fetus[40]. Other studies have shown an increase in the deposition of TG and the expression of FA transport proteins (FAT, FABP3, and FABP4) in the placenta of GDM patients[35]. Treatment of GDM with insulin could significantly increase the phosphorylation of Akt and Erk in the placenta and the expression of placental lipid carriers (FAT, A-FABP, and endothelial lipase) and promote the transfer of placental lipids to the fetus[41]. Several studies have shown that the expression of FAT and TLR4 in the placenta of GDM patients significantly increases and is positively associated with neonatal weight[42]. The ANGPTL3-4-8 axis regulates lipid transport and protein expression and is related to fetal birth weight, body length and placental weight[43]. However, the dysregulated expression of this axis in the placenta of GDM patients has an impact on placental lipid transport and protein expression. These results indicate that an increase in the placental lipid transfer gradient and in the transport of proteins due to an intrauterine high-glucose environment leads to the accumulation of fetal lipids, which may lead to an elevated fetal obesity level and increased neonatal body fat mass in GDM patients.

Although macrosomia is common in the fetuses of GDM patients, growth retardation is a common manifestation in GDM animal models. The pregnancy of STZ-induced GDM rats is characterized by placental enlargement and varying degrees of growth retardation in the offspring[44]. The placental IR pathway is altered by hyperinsulinism and activates the downstream endothelial carbon monoxide synthase to stimulate increased placental angiogenesis[45], thereby affecting placental nutrient metabolism. Animal and *in vitro* experiments have shown that amino acid transport proteins are downregulated in offspring with growth restriction, and the activity of placental mTORC1 is reduced in STZ-induced GDM rat models, resulting in a decrease in L-system amino acid transport proteins in the placenta, which is associated with intrauterine growth restriction and a reduced birth weight[46]. It has also been found in human and *in vitro* experiments that IL-15 is upregulated in the placenta of GDM patients and promotes trophoblast proliferation *in vitro* through the JAK/STAT signaling pathway, which is negatively correlated with neonatal weight[47].

EFFECTS ON THE HORMONE SECRETION FUNCTION OF THE PLACENTA

Animal experiments have shown that the dysregulation of hormones secreted by the placenta during pregnancy may alter insulin signaling and adversely affect fetal growth[11,48]. A number of studies have shown that the level of human placental lactogen (HPL) in GDM patients increases during the third trimester of pregnancy, and the expression levels of HPL in mothers and umbilical cord blood are closely related to placental weight and birth weight[49,50]. Placental enlargement in GDM patients may cause an increase in the levels of growth hormone (GH) and HPL, induce maternal insulin resistance and stimulate the generation of fetal IGF-1 and insulin, thereby resulting in fetal fat deposition and overgrowth[51]. HPL can also regulate fetal growth and development *via* a certain mechanism. A targeted reduction in placental HPL in sheep can lead to early intrauterine growth restriction and a significant decrease in the birth weight of the offspring in the later stage[52]. GH[53] and HPL[54] significantly increased in the placenta of LGA pregnancies, whereas the expression levels of HPL[55] and GH[53] were reduced in small for gestational age pregnancies. These results indicate that placental HPL and GH jointly regulate fetal growth and development in utero.

In addition, insulin/IGF and adipokines secreted by the placenta are also important for fetal growth and development. The expression of the IGF-1-IGFBP-1 axis is dysregulated in the umbilical cord blood of GDM patients, and the opposite changes in IGF-1 and IGFBP-1 expression are observed. The increased bioavailability of IGF-1 caused by a reduction in IGFBP-1 leads to increased glucose uptake and utilization, increasing the risk of macrosomia[56]. Studies have shown a positive correlation between the risk of suffering from GDM and a higher level of IGF-1 in maternal blood[57,58]. Several studies have shown that the expression of IGFBP-1[59], IGFBP-2[58], IGFBP-3[60], and IGFBP-rP1[61] in the umbilical cord blood of GDM patients significantly decreases, leading to a reduction in the ability of IGFBP to bind to IGF-1 and IGF-2[60], whereby the level of free IGF-1 in umbilical cord blood[22] and the phosphorylation of IGF-1R in the placenta increase[21]. These changes improved the signaling activity of free IGF-1 and IGF-2 in umbilical cord blood. Moreover, several studies have shown that the fetal weight of GDM patients is significantly positively correlated with the expression of IGF-1[23,25,62] and IGF-2[63] in the placenta. Cellular experiments and clinical studies have also shown that GDM strengthens placental insulin/IGF-1 signaling, which activates downstream mammalian mTORC1 targets and increases placental nutrient transport[26], leading to fetal overgrowth, as its activation is positively correlated with birth weight[25,64]. These results indicate that changes in the insulin/IGF signaling axis may be an important mechanism for fetal birth weight gain in GDM patients. In addition, GDM patients with macrosomia have higher levels of umbilical cord leptin (LEP) and resistin[65] and lower levels of the maternal adiponectin gene (*ADIPOQ*)[23]. However, the expression levels of LEP and resistin in the umbilical cord were positively correlated with the body weight of large-for-date fetuses, whereas

maternal ADIPOQ was inversely proportional to birth weight[65]. This may be because the phosphate site of IRS-1 was inhibited by low maternal ADIPOQ levels, which, together with insulin/IGF-1/mTOR signaling, regulated nutrients such as glucose, amino acids and lipids to stimulate fetal overgrowth[59,66]. These findings indicate that placental adipokines participate in insulin axis signaling to jointly regulate placental nutrient transport and fetal growth and development.

IMPACTS ON PLACENTAL DNA METHYLATION

It has been reported that the epigenetics of the placenta play key regulatory roles in placental development and function [67]. The impacts of GDM on the global methylation of the placenta and the methylation of imprinted genes and metabolic genes may result in impairments to the placenta and intrauterine fetal development and even an increased susceptibility of the offspring to diseases such as obesity and metabolic syndrome in the later stage (Figure 1).

Global methylation of the placenta and methylation of imprinted genes

A number of studies have shown that the methylation of a large number of genes in the placenta of GDM patients is associated with fetal weight. The differentially methylated position (DMP) of 11 genes in the placenta of GDM patients is associated with birth weight[68]. Among the differentially methylated genes in the placenta of GDM patients, 326 placental genes and 117 umbilical cord genes are also associated with neonatal weight[69].

Studies have shown that three CpG methylation sites in the DNA methylation region of the maternally expressed gene 3 (*MEG3*) on the maternal side of the placenta of GDM patients are significantly increased and are positively correlated with maternal blood glucose and fetal weight, whereas only one CpG position on the fetal side of the placenta is highly methylated and unrelated to fetal weight[70], indicating that maternal metabolic status alters the methylation level of the placenta and participates in fetal development. Some studies have shown that DNA methylation of the maternal imprinted gene mesoderm specific transcript (MEST) in the placenta of GDM patients significantly decreases and is related to GDM, possibly leading to the pathogenesis of GDM macrosomia. Researchers have also found that the methylation of MEST significantly decreases in the peripheral blood of adult obese individuals[71]. These results indicate that MEST is involved in the reprogramming of obesity in offspring and suggest the consequences of placental methylation on early exposure to an adverse intrauterine environment, including the tendency toward obesity in adult offspring. The hypermethylation of the imprinted gene *DLK1* on the fetal and maternal sides of the placenta in GDM patients led to a significant decrease in its gene expression and was positively correlated with fetal weight and maternal two-hour oral glucose tolerance test (OGTT) blood glucose concentration[72], indicating that the methylation of DLK1 may be a potential mechanism for obesity and metabolic programming disorders in childhood and adulthood. Under the influence of a high-glucose environment, IGF-2 and H19, which are also pairs of imprinted genes, exhibit variable methylation levels, and the expression level of IGF-2 increases[73]; moreover, the expression of IGF-2 is directly proportional to the occurrence of macrosomia[63]. Studies have shown that changes in the methylation and expression levels of placental imprinted genes in STZ-induced GDM mice led to the hypomethylation and increased expression level of H19 and the hypermethylation and decreased expression level of PEG3; and the methylation changes of the imprinted genes could be reversed by transferring prokaryotic embryos of diabetic female mice into normal pregnant uteruses[74]. The hypomethylation of the paternally expressed genes IGF1R and IGFBP-3 in the placenta and the high expression of these genes were negatively correlated with maternal blood glucose levels, and the increased expression of IGF1R mRNA was related to the birth weight of newborns, which may be involved in the pathogenesis of GDM macrosomia and increase the susceptibility of offspring to obesity[75]. These experiments indicate that alterations in the methylation of placental imprinted genes and their gene expression levels provide genetic information for fetal adipose tissue and metabolic programming and increase the susceptibility of offspring to metabolic diseases in the later stage.

Methylation of placental metabolic genes

Placental methylation can also affect fetal development by regulating the expression of metabolic genes. Studies have shown that placental LEP, which is capable of regulating insulin signaling and participating in insulin resistance, is associated with the pathogenesis of GDM. The average methylation level of LEP at 23 CpG sites in the placenta of GDM patients was greater than that in the placenta of healthy pregnancies, and LEP, an adipokine for maintaining energy homeostasis, is capable of regulating fetal growth and placental nutrient exchange[76]. However, another study showed that the methylation level of LEP decreased in the placenta of GDM patients, and the DNA hypomethylation level of its gene locus (cg15758240) was negatively correlated with the expression level of LEP (a representative of neonatal obesity) in the fetus at birth and in early childhood obesity[77]. In addition, ADIPOQ and LPL are important metabolic genes in the placenta. The methylation level of ADIPOQ on the maternal side of the placenta is correlated with maternal two-hour OGTT blood glucose concentration, increased insulin resistance, and maternal ADIPOQ levels during pregnancy and after delivery, and higher adiponectin levels in umbilical cord blood are associated with fetal birth weight[78]. Adiponectin is the most abundant circulating hormone secreted by adipocytes and is regulated by the degree of insulin resistance. The methylation of adiponectin may lead to obesity, insulin resistance and glucose metabolic disturbance in offspring and increase the probability of suffering from type 2 diabetes in offspring. The methylation levels of the LPL proximal promoter and intronic CpG islands decreased in the placenta of GDM patients, wherein the hypomethylation levels of LPL at the CpG1 and CpG3 loci were negatively correlated with maternal blood glucose and high-density lipoprotein cholesterol (HDL-C), and the hypomethylation level of the LPL-CpG2 Locus was negatively correlated with the expression level of placental LPL mRNA and HDL-C in umbilical cord blood[79], indicating that the versatility of the methylation levels of LPL may be related to maternal and fetal metabolic profiles and involved in placental lipid transfer

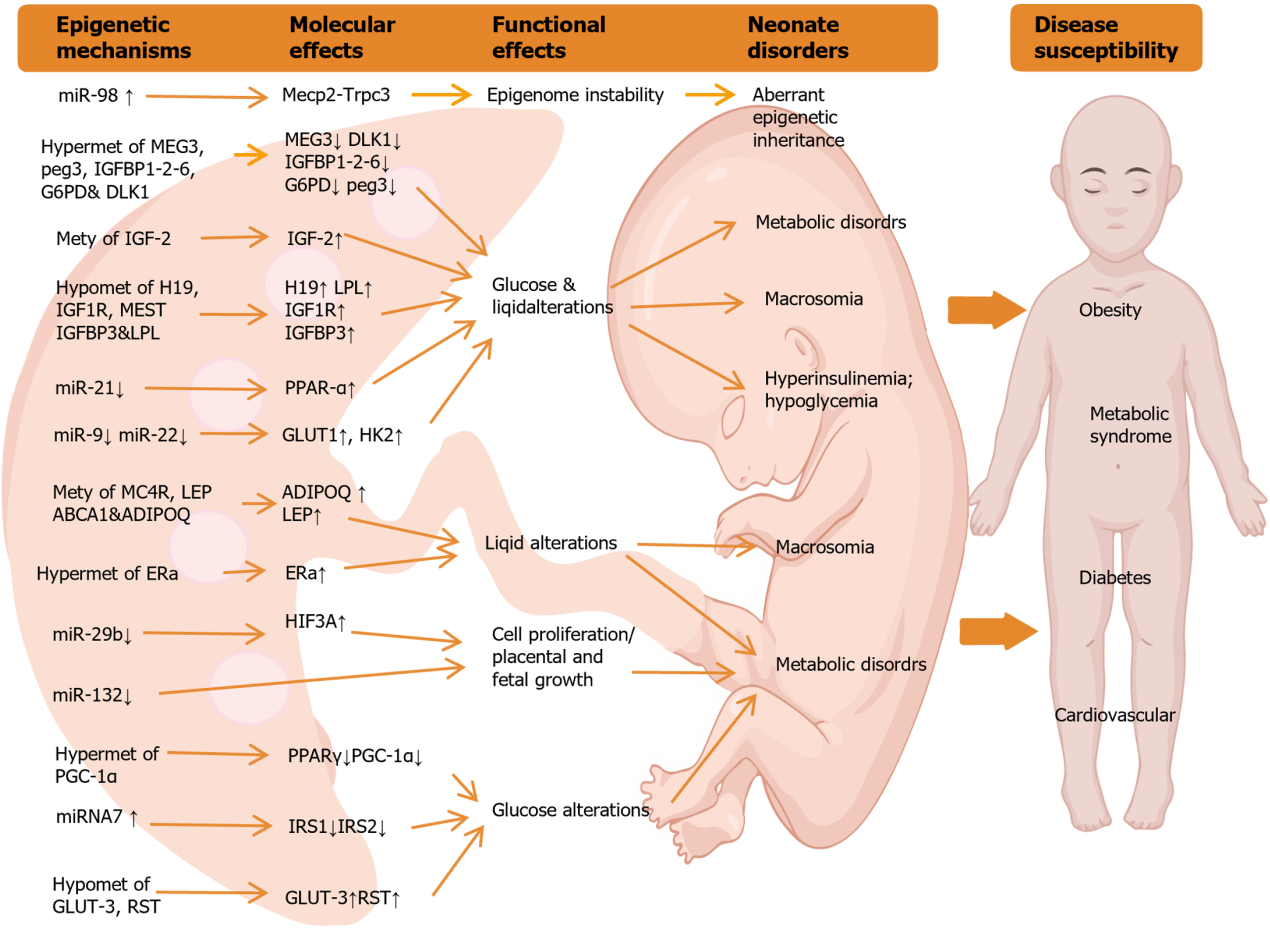

Figure 1 Role of placenta epigenetic modification in offspring development of gestational diabetes mellitus. MEG3: Maternally expressed gene 3; IGF-1R: Insulin-like growth factor 1 receptor; LEP: Umbilical cord leptin; HIF3A: Hypoxia-inducible factor 3 subunit α; PGC-1α: Proliferator-activated receptor-γ coactivator-1α; GLUT: Glucose transporters.

and intrauterine programming of fetal adipose tissue. Another experiment also showed that a low DNA methylation level at the 3.4 CpG site in the placenta of GDM patients was positively correlated with birth weight and mid-childhood fat mass[80]. In addition, the methylation level of LPL in the placenta was inversely proportional to its gene expression, and LPL in placental syncytiotrophoblasts was capable of hydrolyzing TG-rich lipoproteins into FAs to increase maternal-fetal lipid transfer gradients and promote placental lipid transfer to increase fetal weight. These energy metabolism genes participate in the regulation of energy metabolism and insulin sensitivity, and adaptive changes may lead to sustained glucose metabolism disorders in both mothers and offspring.

Studies have shown that an increase in the nuclear receptor estrogen receptor a (ERa) protein and its mRNA level in extracellular trophoblasts on the maternal side of the placenta in GDM patients may be related to hypomethylation of the ERa promoter region[81]. Estrogen secreted by the placenta is an important regulator of fat metabolism and may participate in the programming of fetal fat metabolism in utero. The hypermethylation levels of IGFBP-1, IGFBP-2, IGFBP-6, and G6PD in the placenta of GDM patients were positively correlated with maternal fasting plasma glucose and one-hour blood glucose concentration after an OGTT. The methylation levels of IGFBP and G6PD were negatively correlated with their expression in the placenta. A decreased expression of IGFBP increases the availability of free IGF-1, contributing to the occurrence of macrosomia. Fetal birth weight was significantly negatively correlated with the expression of G6PD mRNA but was positively correlated with methylation[82]. The ATP-binding cassette transporter A1 (ABCA1) is a key regulator for placental lipid transfer. It has been reported that the hypermethylation level of ABCA1 on the maternal side of the placenta in pregnant women with impaired glucose tolerance is correlated with maternal HDL-C levels and two-hour OGTT blood glucose concentrations, and maternal blood glucose and HDL-C act together on the DNA methylation profile of ABCA1; moreover, the hypermethylation level of ABCA1 on the fetal side of the placenta is negatively correlated with TG levels in umbilical cord blood, and the hypomethylation level of ABCA1 in umbilical cord blood is negatively correlated with maternal two-hour OGTT blood glucose concentrations in the second trimester of pregnancy[83]. The difference in the methylation of ABCA1 between the placenta and the umbilical cord may be an adaptive response of the fetus to intrauterine hyperglycemia to compensate for the reduction in the placental transfer of maternal cholesterol, but it also leads to increased susceptibility to dyslipidemia, obesity, impaired endothelial function and cardiovascular diseases in the later stage. The melanocortin 4 receptor (*MC4R*) gene plays a crucial role in regulating metabolism by suppressing appetite and participating in energy control. The methylation levels of the CpG-1 and CpG-2 loci of the energy metabolism gene *MC4R* on the fetal side of the placenta in GDM patients decreased, whereas the

methylation level of the CpG-1 locus of MC4R on the maternal side of the placenta increased in pregnant women with a smoking habit and was related to maternal one-hour and two-hour OGTT glucose concentrations and low-density lipoprotein cholesterol (LDL-C) levels[84]. The spatial difference in the methylation levels of energy metabolism genes between the fetal side and the maternal side of the placenta may be a certain environmental adaptation change to protect the metabolic health of the offspring and reveal the complexity of DNA methylation.

Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a transcriptional coactivator, is a regulator that adjusts nutritional energy homeostasis and metabolism between the placenta and the fetus during pregnancy and participates in the metabolic programming of the fetus. Studies have shown that maternal two-hour OGTT glucose concentrations in the second trimester of pregnancy are positively correlated with DNA hypermethylation at the PGC-1α CpG locus in the placenta, and the degree of correlation increases with maternal weight and insulin resistance during pregnancy and is negatively correlated with the hypomethylation of PGC-1α DNA in the umbilical cord[85]. The difference in methylation between the placenta and the umbilical cord may be an adaptive mechanism to the intrauterine high-glucose environment. In the placenta of GDM patients, the methylation of the PGC-1α promoter increases, the expression of PGC-1α mRNA decreases, and the downregulation of PGC-1α in the placenta is negatively correlated with early fetal blood glucose[86]. The expression of PGC-1α and peroxisome proliferator-activated receptor γ in the placenta of GDM patients decreased and was negatively correlated with that in the offspring during young adulthood[87]. The methylation of PGC-1α may alter the methylation pattern of PGC-1α in fetal endocrine organs (such as islets) and the sensitivity of other tissues to insulin, resulting in an increase in blood glucose and an increased risk of diabetes in offspring. The hypomethylation and increased expression of GLUT-3 and resistin in the placenta of GDM patients led to excessive placental glucose transport to the fetus and increased insulin resistance, thus giving rise to fetal glucose metabolism disorders and macrosomia[88].

In summary, changes in the methylation of placental metabolic genes may underlie the pathogenesis of obesity and other related metabolic diseases. These research data show that epigenetics provides valuable information for the programming of placental and fetal development and can guide future research directions, provide disease prediction information for clinical practice and facilitate the development of prevention and treatment measures.

Transgenerational effects of placental methylation

In the STZ-induced GDM model, intrauterine hyperglycemia may induce hypermethylation of the imprinted gene *Dlk1-DMR* and hypomethylation of IG-DMR and Gtl2-DMR in the placentas of the F1 and F2 generations and affect their gene expression levels, which may result in a reduction in the weight of the placentas of the F1 generation and can be transmitted to the F2 generation through a paternal line[89], indicating that the methylation of key genes in the placenta has potential transgenerational effects on offspring development.

IMPACT ON THE EXPRESSION OF PLACENTAL MIRNAS

The upregulation or downregulation of miRNAs in the placenta can regulate the proliferation and infiltration of placental trophoblasts and thus affect placental development and function. Inactivation of the placental miRNA mechanism has an impact on fetal weight and metabolism and may affect fetal growth and development[90].

An experiment with HTR-8/SVneo and BeWo cells reported that high glucose concentrations inhibited cell viability and reduced the expression levels of placental miR-132, which could promote trophoblast cell proliferation and infiltration[91]. In addition, some studies have reported that placental trophoblast proliferation is related to macrosomia. Human and *in vitro* placental experiments have shown that placental weight is closely related to macrosomia in GDM patients, possibly because Erk1/2 signaling is activated by hyperglycemia and promotes trophoblast cell proliferation [92]. Another study also confirmed that the macrosomia of GDM patients is associated with placental trophoblast proliferation[93]. The expression levels of miR-130b-3p, miR-29a-3p, and miR-let-7a-5p in the placenta of GDM patients decreased with increasing birth weight[94]. MiR-508-3p was upregulated in GDM patients, and EGFR/PI3K/Akt signaling was activated by the targeted reduction in PIKfyve, a negative regulator of EGFR (epidermal growth factor receptor), leading to the occurrence of macrosomia[95]. These findings indicate that the placenta alters key miRNAs involved in fetal development to adapt to a maternal intrauterine hyperglyceemic environment and plays an important role in fetal development.

miRNAs also participate in placental glucose and lipid metabolism. It has been reported that miR-21 is downregulated in the placenta of GDM patients, whereas the expression of PPARα[96], a nuclear receptor involved in lipid and glucose homeostasis, is increased. miR-9 and miR-22 are downregulated in the placenta of GDM patients and upregulate the expression of GLUT1 and HK2, leading to increased glucose uptake in primary syncytiotrophoblasts and HTR8/SVneo cells[97]. The downregulation of miR-29b in the placenta of GDM patients promoted trophoblast activity in the placenta and increased glucose uptake by increasing the expression of hypoxia-inducible factor 3 subunit α (HIF3A)[98]. However, the expression levels of miR-98[99] and miR-199a[100] were significantly increased in the placentas of GDM patients, and these genes indirectly regulated glucose uptake by targeting the Mecp2-Trpc3 pathway. It has been reported that the expression level of miRNA7 in the placenta of GDM patients increases, and the placental insulin signaling pathway and glucose metabolism are regulated by means of targeted downregulation of IRS1 and IRS2[14]. These results indicate that miRNAs participate in glucose metabolism and insulin signaling alterations in the placentas of GDM patients and may be involved in the pathogenesis of GDM and lead to metabolic disorders in offspring. Therefore, the NRS-2002 can also be used as a useful marker for the diagnosis of GDM.

In conclusion, placental epigenetic modifications play an important regulatory role in the programming of fetal development in patients with GDM and are related to maternal metabolism. Multiple placental epigenetic modifications affect fetal development by regulating placental function, gene expression, fetal weight and fetal metabolism. Understanding the relationship between placental epigenetic changes and fetal development is highly important for revealing the molecular mechanism of fetal development and identifying related diseases. In the future, by means of interfering with placental epigenetic abnormalities, new treatments can be explored to improve fetal development and prevent the occurrence of related diseases.

DEFICIENCIES AND PROSPECTS

An intrauterine high-glucose environment alters placental function, epigenetics and gene expression, participates in fetal intrauterine programming, has an important impact on offspring development, and increases the prevalence of obesity, cardiovascular disease and metabolic syndrome in adult offspring. The understanding of the impacts of the placenta on fetal development is insufficient at present, and there is still a long way to go. First, there is a theoretical relationship between placental function and epigenetic abnormalities in GDM patients and fetal development, and there is some supporting evidence to prove their correlation with fetal development. The placenta receives signals from both maternal nutritional reserves and fetal development needs, but the mechanism of integration and the exact nature of these signals and their regulation and influence on a high-glucose environment are still unclear. In the future, further probing of the molecular mechanism and etiology of placental epigenetic changes and their effects on the development of offspring can be performed by means of a molecular pathological epidemiology (MPE)[101] technique, which links potential risk factors with the molecular pathology of diseases and contributes to precision prevention and precision medicine[102], providing a theoretical and scientific basis for early warning, prevention and treatment of GDM. In addition, MPE research can explore the association between GDM and later chronic diseases and other diseases[103], providing new strategies for combined prevention and individualized treatment of diseases. Second, the current research has focused mainly on static analysis of placental function and epigenetics and has lacked observations of dynamic changes. However, the process of fetal development is dynamic, and our study can provide information only on changes at certain time points. To better understand the temporal relationship between placental function and epigenetics and fetal development, long-term follow-up observations are needed to obtain additional comprehensive information. In addition, although the animal models used in the experiments are similar to those used in humans, there are still some differences that prevent direct application of the results to the human placenta. However, further validation combined with human placenta studies is needed.

GDM has a profound impact on the development and subsequent health of offspring. As an intermediary organ between the mother and the fetus, the placenta plays a crucial role, and further investigations of the relationship between MPE changes in the placenta and abnormal fetal development are required to understand the specific mechanism involved in the development of the placenta in offspring and to determine the causal relationship between the placenta and fetal development. Only in this way can we have a deeper understanding of the pathophysiological process of abnormal development in offspring and associate this process with external factors and the development of chronic diseases in the later stage to improve the outcomes of pregnant women with GDM and their offspring.

CONCLUSION

In a word, altered placental function and epigenetic modifications in GDM mothers are associated with an increased risk of obesity, metabolic diseases, and cardiovascular diseases in offspring. These changes may affect the metabolic function of offspring and increase disease susceptibility by changing the expression of genes. Therefore, it is important to understand the impact of changes in placental function and epigenetic modifications of the placenta in GDM on offspring development, and to explore how to optimize maternal and infant health by adjusting placental function and epigenetic modifications. In the future, further efforts should be made to explore the biological mechanism of the placenta and placental MPE, so as to develop more effective prevention and treatment measures to ensure the overall health of GDM patients and their offspring.

FOOTNOTES

Co-first authors: Yan Yi and Tao Wang.

Co-corresponding authors: San-Hong Zhang and Wei Xu.

Author contributions: Yi Y and Wang T reviewed and summarized the literature and wrote the paper; Zhang SH and Xu W designed and revised the manuscript; Xu W is the guarantor of this work; all authors were involved in the critical review of the results and have contributed to read and approved the final manuscript; Yi Y and Wang T contributed equally to this work as co-first authors, Zhang SH and Xu W as co-corresponding authors. The reasons for designating Zhang SH and Xu W as co-correspondent authors are threefold. First, the research was performed as a collaborative effort, and the designation of co-corresponding authorship accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the study and the resultant paper.

This also ensures effective communication and management of post-submission matters, ultimately enhancing the paper's quality and reliability. Second, the overall research team encompassed authors with a variety of expertise and skills from different fields, and the designation of co-corresponding authors best reflects this diversity. This also promotes the most comprehensive and in-depth examination of the research topic, ultimately enriching readers' understanding by offering various expert perspectives. Third, Yi Y and Wang T contributed efforts of equal substance throughout the research process. The choice of these researchers as co-first authors acknowledges and respects this equal contribution, while recognizing the spirit of teamwork and collaboration of this study.

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MINIREVIEWS

Roles of fibroblast growth factors in the treatment of diabetes

Chun-Ye Zhang, Ming Yang

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Abstract

Diabetes affects about 422 million people worldwide, causing 1.5 million deaths each year. However, the incidence of diabetes is increasing, including several types of diabetes. Type 1 diabetes (5%-10% of diabetic cases) and type 2 diabetes (90%-95% of diabetic cases) are the main types of diabetes in the clinic. Accumulating evidence shows that the fibroblast growth factor (FGF) family plays important roles in many metabolic disorders, including type 1 and type 2 diabetes. FGF consists of 23 family members (FGF-1-23) in humans. Here, we review current findings of FGFs in the treatment of diabetes and management of diabetic complications. Some FGFs (*e.g.*, FGF-15, FGF-19, and FGF-21) have been broadly investigated in preclinical studies for the diagnosis and treatment of diabetes, and their therapeutic roles in diabetes are currently under investigation in clinical trials. Overall, the roles of FGFs in diabetes and diabetic complications are involved in numerous processes. First, FGF intervention can prevent high-fat diet-induced obesity and insulin resistance and reduce the levels of fasting blood glucose and triglycerides by regulating lipolysis in adipose tissues and hepatic glucose production. Second, modulation of FGF expression can inhibit renal and cardiac fibrosis by regulating the expression of extracellular matrix components, promote diabetic wound healing process and bone repair, and inhibit cancer cell proliferation and migration. Finally, FGFs can regulate the activation of glucose-excited neurons and the expression of thermogenic genes.

Key Words: Fibroblast growth factors; Type 1 diabetes; Type 2 diabetes; Metabolic disorders; Treatment; Clinical trials

Core Tip: Diabetes affects about 422 million people worldwide, causing 1.5 million deaths each year. However, the incidence of diabetes is increasing, including both type 1 and type 2 diabetes. New therapies are needed to treat diabetes and manage its complications. The fibroblast growth factor (FGF) family members play important roles in many metabolic disorders, including diabetes. To date, a total of 23 family members (FGF1-23) have been found in humans. Some FGFs, such as FGF-15, FGF-19, and FGF-21, have antidiabetic functions in preclinical studies, and they are under investigation in clinical trials for examining the therapeutic effects in patients.

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder that affects different ages of people by inducing abnormal levels of blood sugar in the body. According to the report on the official website of the World Health Organization (WHO, https://www.who.int/, accessed on October 26, 2023), there are about 422 million people with diabetes worldwide, and 1.5 million deaths are directly caused by diabetes each year. The incidence of diabetes is increasing[1,2]. There are several types of diabetes. Type 1 DM (T1DM, 5%-10% of diabetic cases) and type 2 DM (T2DM, 90%-95% of diabetic cases) are the main types of diabetes in the clinic[3]. T1DM occurs when the insulin-producing pancreatic beta cells are damaged by factors such as autoimmune attack[4], while T2DM is characterized by both insulin resistance and beta cell dysfunction that cause persistent hyperglycemia[5]. As reported by the WHO, diabetes and diabetes-related kidney disease caused about 2 million deaths in 2019. Therefore, new therapies are urgently needed to treat diabetes and diabetes-related complications.

Fibroblast growth factors (FGFs), consisting of 23 family members (FGF-1-23) in humans[6,7], play important roles in metabolic homeostasis and cell biological processes since alteration of the expression of FGFs is implicated in many chronic diseases. These diseases include obesity[8,9], metabolic-associated fatty liver disease[10-13], diabetes[14,15], and diabetic complications such as hyperthyroidism[16], chronic kidney disease (CKD)[17,18], cardiovascular disease[19,20], and cancer[21,22]. Investigations have shown that FGFs can function as molecular targets for the treatment of diabetes and diabetes-associated metabolic disorders.

In this mini-review, we first review the roles of FGFs in the pathogenesis of diabetes and diabetic complications, and then we briefly summarize the findings of clinical trials regarding the functions of FGFs in the treatment of diabetes and metabolic disorders.

FGFS PLAY AN IMPORTANT ROLE IN DIABETES AND RELATED DISEASES

Of the recognized 23 FGFs, some have been extensively investigated such as FGF-21 in diabetes, and others have not been well studied such as FGF-8. Although the same family of FGFs has similar principle functions, the functions of each member remain distinct in diabetes. Therefore, the following section will briefly introduce the function of each member and mainly focus on the function related to metabolic syndrome, diabetes, and diabetic complications.

FGF-1

FGF-1 can be produced by adipose tissue to regulate glucose uptake by modulating the glucose transporters (GLUTs), GLUT1 and GLUT4[23]. FGF-1 also inhibits lipolysis in adipose tissues to suppress the production of free fatty acids (FFAs) that transport into the liver to produce hepatic glucose. Mechanistically, FGF-1 binds to its FGF receptor 1 (FGFR1) to activate the phosphorylation of phosphodiesterase 4D to inhibit lipolysis in adipocytes by inhibiting cyclic adenosine monophosphate-protein kinase A axis[24]. A single parenteral treatment of recombinant FGF-1 can reduce glucose levels in diabetic ob/ob mice and diet-induced obese (DIO) mice that mimic human T2DM[25]. In summary, FGF-1 displays anti-obesity and antidiabetic function by regulating glucose transport, FFA production in obese tissues, and glucose production in the liver.

FGF-2

The binding of FGF-2 to its receptor FGFR can activate intracellular mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 to increase intramuscular adipogenesis in the aged human skeletal muscle, by increasing the phosphorylation of Fos-related antigen and microRNA-29a (miR-29a) expression levels[26]. In mice with diabetic nephropathy, Klotho (a co-receptor for FGF-23) can inhibit renal injury and fibrosis by suppressing FGF-2 expression that is negatively associated with E-cadherin expression[27]. However, FGF-2 isoforms may play different roles in diabetic nephropathy in genetically diabetic db/db mice, with the upregulation of low molecular weight FGF-2 expression and downregulation of high molecular weight FGF-2 expression in the kidney[28]. Thus, FGF-2 may have an unfavorable role in diabetes and relative diseases.

FGF-3

One study showed that there is insulin-dependent diabetes mellitus locus on chromosome 11q13 (IDDM4), which is located near the FGF-3 locus[29]. In addition, FGF-3 and its receptor are downregulated in diabetic retinopathy[30,31]. However, the specific role of FGF-3 in diabetes remains unknown and needs to be further investigated.

FGF-4

The expression levels of FGF-4 and FGFR-2 are increased in the embryo of female BALB/c mice with diabetes compared to their expression levels in the embryo of non-diabetic control mice[32], suggesting their roles in embryo development in maternal diabetes. Administration of FGF-4 *via* intracerebroventricular injection shows an antidiabetic function in male db/db mice and DIO mice by activating glucose-excited neurons *via* FGFR1 and deactivating glucose-inhibited neurons [33]. These studies suggest that the roles of FGF-4 in diabetes may be different in embryo development and postnatal.

FGF-5

FGF-5 can regulate the apoptosis and proinflammation of retinal ganglion cells in diabetic retinopathy by upregulating the expression of cytokines such as tumor necrosis factor-α and interleukin-6[34]. This study also showed that the expression of FGF-5 can be regulated by miR-145-5p, functioning as a potential treatment option. Long non-coding RNA (lncRNA) taurine up-regulated gene 1 (TUG1) expression was downregulated in the islets of mice with a high-fat diet (HFD) compared to that in mice fed a normal diet. Knockdown of lncRNA TUG1 can inhibit glucose-induced proliferation of islet cell line MIN6 cells and promote cell apoptosis by increasing the expression of miR-188-3p to suppress the expression of FGF-5[35]. Overall, FGF-5 shows anti-apoptotic function in obesity and diabetic retinopathy, which can be regulated by non-coding RNAs.

FGF-6

The expression levels of FGF-6 and FGF-9 in adipose tissues can be induced by thermogenic factors such as exposure to cold and exercise, and these two FGFs can upregulate the expression of uncoupling protein-1 (UCP1) in brown and white preadipocytes by activating FGFR3[36]. Overexpression of FGF-6 in inguinal white adipose tissues can inhibit HFD–induced obesity and insulin resistance in lean mice. Mechanistically, FGF-6 functions as an autocrine or paracrine factor to promote platelet-derived growth factor receptor alpha-expressing adipocyte progenitor cell proliferation by regulating the extracellular signal–regulated kinase signaling pathway[37]. Another study showed that overexpression of FGF-6 in mouse skeletal muscle tissues can suppress HFD-induced insulin resistance and body weight increase[38]. In summary, overexpression of FGF-6 can inhibit HFD-induced insulin resistance in obese subjects.

FGF-7

Treatment with FGF-7-loaded galactosylated poly (DL-lactide-co-glycolic acid) particles can improve the islet engraftment into the liver and normalize blood glucose levels in mice with diabetes[39]. In addition, FGFs play a key role in the diabetic wound healing process[40]. For example, one study reveals that inhibition of miR-155 can restore FGF-7 expression to improve diabetic wound healing and reduce wound inflammation[41]. In summary, FGF-7 has diverse roles in diabetic subjects by reducing glucose levels and improving wound healing.

FGF-8

FGF-8 plays a key role in brain development and neuron differentiation by interacting with its receptors such as FGFR1 [42]. However, the specific role of FGF-8 in T2DM and its relative metabolic disorders remains to be studied.

FGF-9

The expression of FGF-9 is increased in the subcutaneous white adipose tissues in obese humans and mice, which can inhibit thermogenic gene expression to activate the hypoxia-inducible factor (HIF) pathway to regulate the adipose browning process[43]. Like FGF-6, FGF-9 can induce the expression of UCP1 in adipocytes and preadipocytes *via* binding with FGFR3 to regulate systemic energy metabolism[36]. Another study demonstrated that the expression of FGF-9 is increased in patients with nonalcoholic steatohepatitis-associated hepatocellular carcinoma (HCC), which promotes the expression of extracellular matrix components by regulating the β-catenin signaling pathway[44]. Therefore, the function of FGF-9 is tissue-dependent.

FGF-10

FGF-10 and its receptor FGFR2b are involved in the development of the digestive system, including the pancreas[45]. FGF-10 is required for the development of the pancreas during early organogenesis[46,47]. As an angiogenic factor, FGF-10 expression is upregulated in epididymal white adipose tissue, endothelial cells, and preadipocytes in HIF-1α deficient mice[48].

FGF-11

FGF-11 functions differently in adipocytes and other cells. FGF-11, a master mediator of adipogenesis, can inhibit adipocyte differentiation by regulating the expression of peroxisome proliferator-activated receptor gamma (PPARγ). By contrast, the PPARγ agonist rosiglitazone can restore adipogenesis, which is suppressed by knockdown of the gene *FGF11*[49]. Knockdown of *FGF11* can significantly reduce mesangial cell proliferation and fibrosis in the progression of diabetic nephropathy[50]. Silencing *FGF11* in the mouse hypothalamus can reduce HFD-induced body weight gain and

fat accumulation by increasing brown adipose tissue thermogenesis and insulin intolerance[51]. In addition, FGF-11 regulates the differentiation and thermogenesis of brown adipocytes in goats[52].

FGF-12

The role of FGF-12 is mainly investigated in cardiovascular disease. FGF-12 upregulation can improve cardiac dysfunction in mice with myocardial infarction by reducing the production of extracellular matrix components in cardiac fibroblasts induced by angiotensin II, including fibronectin and collagens I and III[53]. It also plays an important role in vascular remodeling by regulating the phenotypic change of vascular smooth muscle cells[54].

FGF-13

The serum level of FGF-13 was decreased in patients with impaired glucose tolerance and T2DM compared to that in the healthy controls, suggesting that it could serve as a diagnostic marker for T2DM[55]. In addition, FGF-13 plays an important role in diabetic nephropathy[56] and obesity[57]. However, the function of FGF-13 in glucose regulation and T2DM remains to be studied.

FGF-14

Currently, the effects of FGF-14 are broadly investigated in tumors. FGF-14 is downregulated in lung adenocarcinomas [58], playing a pivotal role in cancer cell proliferation and migration. Overexpression of FGF-14 is associated with a better overall survival of pancreatic ductal adenocarcinoma patients[59].

FGF-15

Mouse FGF-15 is the homolog of human FGF-19. Overexpression of mouse FGF-15 or administration of recombinant human FGF-19 can decrease the levels of fasting blood glucose, FFAs, and triglycerides, and homeostasis model assessment of insulin resistance cores in pregnant mice with HFD compared to corresponding control mice[60]. The antidiabetic effects of total flavonoids extracted from tea are mediated by activation of the farnesoid X receptor/FGF-15 axis[61]. Another study also showed that FGF-15/FGF-19 treatment can inhibit hepatic lipogenesis in mice by activating small heterodimer partner and DNA methyltransferase-3a[62]. Overall, FGF-15 displays antidiabetic function by reducing the levels of fasting blood glucose, FFAs, insulin resistance, and hepatic lipogenesis.

FGF-16

FGF-16 is a target of microRNAs, such as miR-372-3p and miR-144-3p, which can regulate high glucose-induced glomerular endothelial cell dysfunction in patients with diabetic retinopathy[63] and suppress high-glucose-induced proliferation of human umbilical vein endothelial cells and human retinal endothelial cells to potentially suppress diabetic retinopathy[64]. Another study also showed that FGF-16 can be regulated by miR-520b to regulate lung cancer cell proliferation[65]. In summary, FGF-16 regulates cell dysfunction and proliferation in diabetes and cancers.

FGF-17

The function of FGF-17 has been investigated in cancers. FGF-17 has been shown to function as a potent diagnostic marker for acute myeloid leukemia[66]. As a subfamily member of FGF-8, it has been detected to be upregulated in 59% of human HCC samples to contribute to angiogenesis and cancer cell survival[67]. The roles of FGF-17 in diabetes are less studied.

FGF-18

FGF-18 plays multiple roles in many diseases including bone repair[68], diabetic wound healing[69], and cancer[70-72]. A recent study showed that the expression of FGF-18 is associated with liver fibrosis in human liver tissues, which can promote liver fibrosis in mouse models[73]. However, the specific role of FGF-18 in diabetes remains to be studied.

FGF-19

Intracerebroventricular injection of recombinant FGF-1 or FGF-19 can induce a 60% reduction of glucose production in the livers of mice with T1DM, as well as lipolysis in the body[74]. A clinical trial study finds that circulating serum levels of FGF-19 are significantly decreased in obese patients independent of insulin resistance[75]. Another study also reveals that serum levels of FGF-19 are significantly decreased in patients with T2DM and metabolic syndrome compared to healthy controls[76]. Low serum level of FGF-19 is positively associated with T1DM as a contributing factor, which is negatively associated with the levels of fasting blood glucose[77]. These results suggest that FGF-19 can regulate the levels of glucose to ameliorate insulin resistance and diabetes.

FGF-20

FGF-20 has favorable roles in several chronic diseases. For example, FGF-20 plays a protective role in cardiac hypertrophy by activating silent information regulator 1 to inhibit oxidative stress-induced myocardial injury[78]. Increased plasma FGF-20 protein can delay the progression of diabetic renal diseases at the end stage[79]. In addition, rs12720208 polymorphism in the gene *FGF20* has been found to be associated with the susceptibility of Parkinson's disease[80]. The function of FGF-20 in diabetes remains unclear.

FGF-21

The expression level of FGF-21 has been found to be positively associated with the risk of T2DM in a cross-sectional study in the southern part of China, serving as a potential diagnostic marker[81]. Treatment with recombinant human FGF-21 can ameliorate insulin resistance, hyperglycemia, and endothelial dysfunction in T2DM mice induced by HFD-streptozotocin treatment by activating the calcium/calmodulin-dependent protein kinase kinase 2/AMP-activated protein kinase alpha signaling pathway[82]. FGF-21 as a peptide hormone plays beneficial effects on weight loss, glucose and fatty acid metabolism, and inflammation[83].

FGF-22

FGF-22 plays an essential role in the recovery process of spinal cord injury, which can inhibit endoplasmic reticulum stress-induced apoptosis[84,85]. The rs8109113 polymorphism of the gene *FGF22* has been shown to be associated with hypertension and height[86]. Currently, the function of FGF-22 remains under further investigation.

FGF-23

FGF-23 plays an important role in maintaining serum phosphate concentration in CKD. Patients with diabetic kidney disease received a high-phosphate diet at a daily dose of 1800 mg for 6 d had an increased serum FGF-23 at the first 3 d from baseline, but had a trend to decrease after day 3, whereas this diet steadily increased the level of FGF-23 in non-diabetic patients[87]. Ramipril, an angiotensin-converting enzyme inhibitor, is commonly applied to treat hypertension, heart failure, and diabetic kidney disease. Ramipril treatment significantly decreases serum FGF-23 levels, resulting in improvement in proteinuria and an endothelium-dependent flow-mediated response to ischemia in patients with T2DM and stage 1 CKD[88]. Overall, FGFs exhibit diverse and different roles in diabetes and the associated diseases (Table 1), and targeting some FGFs (*e.g.*, FGF-15, FGF-19, and FGF-21) may facilitate the treatment of diabetes.

POTENTIAL ROLES OF FGF IN DIABETES AND DIABETIC COMPLICATIONS IN CLINICALS

In this section, we briefly introduce several clinical trials about the roles of FGF in diabetes and diabetic complications. Several trials (https://clinicaltrials.gov, numbers including NCT02667964, NCT01858597 or NCT03816605, NCT00491322, NCT04012983, and NCT05937737) have been performed to investigate the roles of FGFs in insulin secretion, insulin resistance, regulation in the expression of insulin receptor substrate 1 and glucose transporter 1 in gestational diabetes mellitus (GDM), and function as biomarkers for periodontal disease in patients with diabetes, as well as the association of FGF expression levels with the intake of phytochemicals in diet and dietary total antioxidant capacity in patients with T2DM.

The impact of physical activity and diet intake on FGF expression in DM patients has been investigated. For example, the relationship between FGF-21 expression and physical activity in regulation of insulin secretion in patients with T1DM or T2DM, and healthy volunteers was investigated in a trial (NCT02667964). Another trial (NCT05937737) investigated the impact of phytochemical intake from the diet and total dietary antioxidant capacity measured by different methods on the expression of serum FGF-21 in patients with T2DM. Given the regulatory effect of vitamin D on insulin secretion in the pancreas, ergocalciferol (vitamin D2) was applied to treat vitamin D deficiency-related insulin resistance and regulate FGF-23 expression in patients (NCT00491322). In addition, the functions of FGFs have been investigated in diabetic complications. GDM is the most common complication in pregnant women. The roles of FGF-19 and FGF-21 in regulating insulin resistance, dyslipidemia, and glucose intolerance in GDM (NCT01858597 and NCT03816605), due to their effects on the expression of insulin receptor substrate-1 and glucose transporter-1 in placenta. Moreover, an observational study (NCT04012983) was conducted to investigate the diagnostic role of FGF-21 from gingival crevicular fluid in periodontal disease in diabetic and nondiabetic patients, in combination with an adipokine chemerin. However, the therapeutic roles of FGF in diabetes remain unknown. More clinical trials are expected to validate pre-clinical findings of FGFs such as FGF-19 and FGF-21 in diabetes.

CONCLUSION

In this minireview, the roles of FGFs in diabetes and other related diseases, such as metabolic syndrome, wound healing, and cancers in current studies are reviewed. The beneficial functions of FGFs in diabetes and diabetic complications comprise suppression of hepatic glucose production and lipolysis in adipose tissues, reduction of levels of fasting blood glucose and triglycerides, inhibition of renal injury and fibrosis, inhibition of HFD-induced obesity and insulin resistance, inhibition of cancer cell proliferation and migration, and promotion of diabetic wound healing process and bone repair (Figure 1). In addition, FGFs can regulate the activation of glucose-excited neurons, the expression of thermogenic genes, and the production of extracellular matrix components in cardiac fibroblasts. Although there are 23 FGF family members, only some FGFs such as FGF-15, FGF-19, and FGF-21 have been broadly investigated in cell and animal models for diabetic disease treatments. The functions of most FGFs in diabetes remain less studied. Moreover, only some clinical trials have been performed to investigate the roles of FGF in insulin secretion, insulin resistance, regulation in the expression of insulin receptor substrate 1 and glucose transporter 1 in gestational diabetes mellitus, function as biomarkers for periodontal disease in patients with diabetes, as well as their expression levels with the association of dietary total antioxidant capacity in patients with T2DM. Therefore, more clinical trials are waited to validate preclinical

Table 1 The effects of fibroblast growth factors on diabetes and diabetes-associated diseases

Diabetes	FGFs	Functions	Ref.
Type 2 diabetes	FGF-1	A single parenteral treatment of recombinant FGF-1 can decrease glucose levels in diabetic ob/ob mice and DIO mice that mimic human type 2 diabetes	Suh *et al*[25]
Diabetic nephropathy	FGF-2	In mice with diabetic nephropathy, Klotho (a co-receptor for FGF-23) can inhibit renal injury and fibrosis by suppressing FGF-2 expression that is negatively associated with E-cadherin expression	Dong *et al*[27]
Diabetic retinopathy	FGF-3	FGF-3 and its receptor have been found to be downregulated in diabetic retinopathy	Ljubimov *et al*[30], Saghizadeh *et al*[31]
Type 2 diabetes	FGF-4	Intracerebroventricular administration of FGF-4 shows an anti-diabetic function in male db/db mice and DIO mice by activating glucose-excited neurons *via* FGFR1, while it can also deactivate glucose-inhibited neurons	Sun *et al*[33]
Type 2 diabetes	FGF-5	Knockdown of lncRNA TUG1 can inhibit glucose-induced proliferation of islet cell line MIN6 cells and promote cell apoptosis by increasing expression miR-188-3p to suppress the expression of FGF-5	Zhang *et al*[35]
Obesity and insulin resistance	FGF-6	Overexpression of FGF-6 in inguinal white adipose tissue can inhibit HFD–induced obesity and insulin resistance in lean mice, while overexpression of FGF-6 in mouse skeletal muscle tissues can also suppress HFD-induced insulin resistance and bodyweight increase	Liu *et al*[37], Xu *et al*[38]
Type 1 diabetes	FGF-7	Treatment with FGF-7-loaded galactosylated poly (DL-lactide-co-glycolic acid) particles can improve the islet engraftment into the liver and normalize blood glucose levels in mice with diabetes	Alwahsh *et al*[39]
Neuron differentiation	FGF-8	FGF-8 plays a key role in brain development and neuron differentiation by interacting with its receptors such as FGFR1	Yellapragada *et al*[42]
Non-alcoholic steatohepatitis (NASH)-associated hepatocellular carcinoma (HCC)	FGF-9	A study also shows that the expression of FGF-9 was increased in patients with NASH-HCC, which regulated the expression of extracellular matrix components by regulating the β-catenin signaling pathway	Zhang *et al*[44]
Pancreas organogenesis	FGF-10	FGF-10 is required for the development of the pancreas during early organogenesis	Bhushan *et al*[46], Norgaard *et al*[47]
Diabetic nephropathy	FGF-11	FGF-11 knockdown can significantly reduce mesangial cell proliferation and fibrosis in the progression of diabetic nephropathy	Liu *et al*[50]
Cardiac dysfunction	FGF-12	FGF-12 upregulation can improve cardiac dysfunction in mice with myocardial infarction by reducing the production of extracellular matrix components in cardiac fibroblasts induced by angiotensin II, including fibronectin and collagen I and III	Liu *et al*[53]
Type 2 diabetes	FGF-13	The serum level of FGF-13 was decreased in patients with impaired glucose tolerance and T2DM compared to that in the healthy controls, suggesting that it could serve as a diagnostic marker for T2DM	Che *et al*[55]
Cancers	FGF-14	FGF-14 plays a pivotal role in cancer progression and prognosis	Turkowski *et al*[58], Raja *et al*[59]
Diabetes, obesity, insulin resistance, and non-alcoholic fatty liver disease	FGF-15	Mouse FGF-15 is the homolog of human FGF-19. Overexpression or activation of FGF-15 or FGF-19 can decrease the levels of fasting blood glucose, free fatty acids, triglycerides, and insulin resistance, which also displays anti-diabetic effects and inhibits hepatic lipogenesis	Zhao *et al*[60], Hu *et al*[61], Kim *et al*[62]
Diabetic nephropathy and diabetic retinopathy	FGF-16	FGF-16 is a target of microRNAs such as miR-372-3p and miR-144-3p, which can regulate high glucose-induced glomerular endothelial cell dysfunction in patients with diabetic nephropathy and suppress high-glucose-induced proliferation of human umbilical vein endothelial cells and human retinal endothelial cells to potentially suppress diabetic retinopathy	Meng *et al*[63], Chen *et al*[64]
Cancers	FGF-17	FGF-17 plays a key role in cancer diagnosis (*e.g.*, acute myeloid leukemia) and cancer cell survival (*e.g.*, hepatocellular carcinoma)	Ling and Du[66], Gauglhofer *et al*[67]
Liver fibrosis	FGF-18	Overexpression of FGF-18 in mouse liver can promote liver fibrosis development	Tsuchiya *et al*[73]
Type 1 and type 2 diabetes	FGF-19	Serum levels of FGF-19 were significantly decreased in patients with T1DM and T2DM	Barutcuoglu *et al*[76], Hu *et al*[77]
Diabetic renal diseases	FGF-20	Increased plasma FGF-20 protein could delay the progression of diabetic renal diseases at the end stage	Md Dom *et al*[79]
Type 2 diabetes	FGF-21	Treatment with recombinant human FGF-21 can ameliorate insulin resistance, hyperglycemia, and endothelial dysfunction in T2DM mice induced by HFD-STZ treatment by activating the CaMKK2/AMP-AMPKα signaling pathway	Ying *et al*[82]
Spinal cord injury	FGF-	FGF-22 plays an essential role in the recovery process of spinal cord injury, which can inhibit	Aljović *et al*[84],

	22	endoplasmic reticulum stress-induced apoptosis	Zhu *et al*[85]
Diabetic nephropathy	FGF-23	FGF-23 could be implicated in proteinuria and endothelial dysfunction in patients with diabetic nephropathy	Yilmaz *et al*[88]

DIO: Diet-induced obese; HCC: Hepatocellular carcinoma; HFD: High-fat diet; lncRNA: Long non-coding RNA; NASH: Nonalcoholic steatohepatitis; STZ: Streptozotocin.

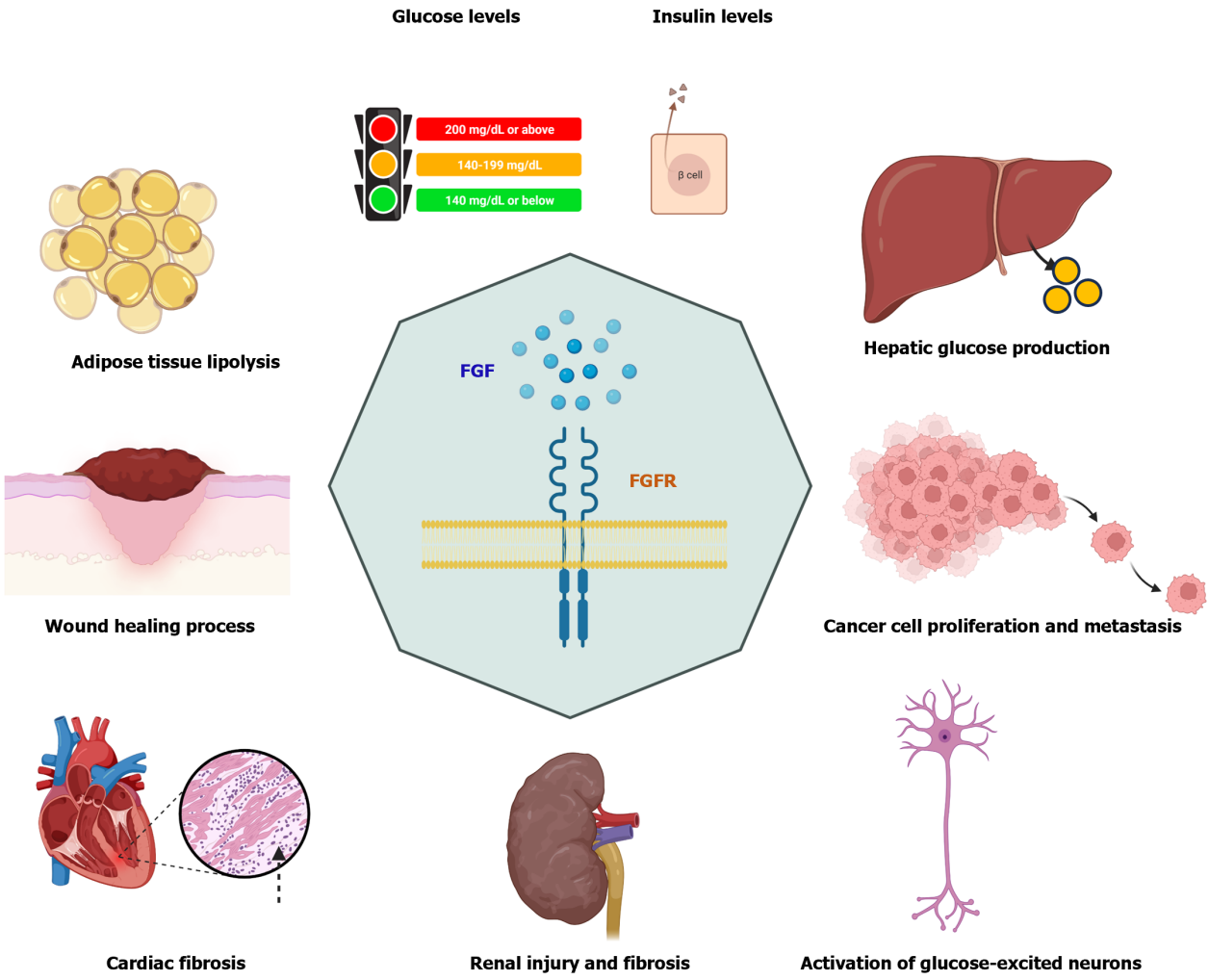

Figure 1 Functions of fibroblast growth factor/fibroblast growth factor receptor signaling pathways in diabetes and diabetes-relative diseases. The mechanisms of action of fibroblast growth factors (FGFs) include suppression of hepatic glucose production and lipolysis in adipose tissues, activation of glucose-excited neurons, inhibition of renal injury and fibrosis, inhibition of insulin resistance, regulation of extracellular matrix components in cardiac fibroblasts, inhibition of cancer cell proliferation and migration, reduction of levels of fasting blood glucose and triglycerides, and promotion of the diabetic wound healing process. FGFR: Fibroblast growth factor receptor. All cartoons in this figure were prepared using Biorender.

findings of the roles of FGF in diabetes and investigate new drugs or small molecules targeting FGFs to treat diabetes and diabetes-related metabolic disorders.

FOOTNOTES

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ORIGINAL ARTICLE

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Associations between Geriatric Nutrition Risk Index, bone mineral density and body composition in type 2 diabetes patients

Xiao-Xiao Zhu, Kai-Feng Yao, Hai-Yan Huang, Li-Hua Wang

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Abstract

BACKGROUND

Type 2 diabetes mellitus (T2DM), a fast-growing issue in public health, is one of the most common chronic metabolic disorders in older individuals. Osteoporosis and sarcopenia are highly prevalent in T2DM patients and may result in fractures and disabilities. In people with T2DM, the association between nutrition, sarcopenia, and osteoporosis has rarely been explored.

AIM

To evaluate the connections among nutrition, bone mineral density (BMD) and body composition in patients with T2DM.

METHODS

We enrolled 689 patients with T2DM for this cross-sectional study. All patients underwent dual energy X-ray absorptiometry (DXA) examination and were categorized according to baseline Geriatric Nutritional Risk Index (GNRI) values calculated from serum albumin levels and body weight. The GNRI was used to evaluate nutritional status, and DXA was used to investigate BMD and body composition. Multivariate forward linear regression analysis was used to identify the factors associated with BMD and skeletal muscle mass index.

RESULTS

Of the total patients, 394 were men and 295 were women. Compared with patients in tertile 1, those in tertile 3 who had a high GNRI tended to be younger and had lower HbA1c, higher BMD at all bone sites, and higher appendicular skeletal

muscle index (ASMI). These important trends persisted even when the patients were divided into younger and older subgroups. The GNRI was positively related to ASMI (men: $r = 0.644$, $P < 0.001$; women: $r = 0.649$, $P < 0.001$), total body fat (men: $r = 0.453$, $P < 0.001$; women: $r = 0.557$, $P < 0.001$), BMD at all bone sites, lumbar spine (L1-L4) BMD (men: $r = 0.110$, $P = 0.029$; women: $r = 0.256$, $P < 0.001$), FN-BMD (men: $r = 0.293$, $P < 0.001$; women: $r = 0.273$, $P < 0.001$), and hip BMD (men: $r = 0.358$, $P < 0.001$; women: $r = 0.377$, $P < 0.001$). After adjustment for other clinical parameters, the GNRI was still significantly associated with BMD at the lumbar spine and femoral neck. Additionally, a low lean mass index and higher β-collagen special sequence were associated with low BMD at all bone sites. Age was negatively correlated with ASMI, whereas weight was positively correlated with ASMI.

CONCLUSION

Poor nutrition, as indicated by a low GNRI, was associated with low levels of ASMI and BMD at all bone sites in T2DM patients. Using the GNRI to evaluate nutritional status and using DXA to investigate body composition in patients with T2DM is of value in assessing bone health and physical performance.

Key Words: Geriatric Nutrition Risk Index; Bone mineral density; Skeletal muscle mass; Type 2 diabetes

Core Tip: Osteoporosis and sarcopenia are highly prevalent in type 2 diabetes mellitus (T2DM) patients. In people with T2DM, the association between nutrition, sarcopenia, and osteoporosis has rarely been explored. We observed that poor nutrition, as indicated by a low Geriatric Nutritional Risk Index (GNRI), was associated with low levels of ASMI and bone mineral density at all bone sites in T2DM patients. Using the GNRI to evaluate nutritional status and using dual energy X-ray absorptiometry to investigate body composition in patients with T2DM is of value in assessing bone health and physical performance.

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INTRODUCTION

Over the past few years, there has been a rise in the prevalence of osteoporosis and sarcopenia among the elderly population, leading to physical impairment, diminished quality of life and even death of patients[1,2]. Type 2 diabetes mellitus (T2DM), a rapidly growing public health problem, is one of the most common chronic metabolic disorders in older individuals[3]. For patients with T2DM, osteoporosis is one of the possible long-term complications[4]. Sarcopenia, or loss of muscle mass and function, is a major cause of disability in diabetes[5]. Therefore, it is imperative to identify early sarcopenia, osteoporosis and their risk factors in older individuals with T2DM. Subsequently, suitable measures should be taken to avert and manage this ailment.

As a multifactorial systemic disease, many factors contribute to sarcopenia, such as age, sex, body mass index (BMI), duration of diabetes, glycemic control, nutritional status, and lifestyle[6-8]. Sarcopenia is commonly believed to be a decline in skeletal muscle mass and reduced muscle function that occurs with age. In sarcopenia research, the Asia Working Group for Sarcopenia suggests the utilization of the skeletal muscle index (SMI). This index is calculated by dividing the appendicular skeletal muscle mass (ASMM) by the square of height, providing an adjusted measurement of muscle mass[9]. The factors associated with osteoporosis in T2DM include age, sex, BMI, serum vitamin D concentrations, lifestyle factors, duration of diabetes[10], and nutritional risk[11]. Since there are several common factors in osteoporosis and sarcopenia, many studies of the association between osteoporosis and skeletal muscle mass have been reported. The connection between low muscle mass and osteoporosis in patients with T2DM remains uncertain.

Malnutrition is frequently found in elderly individuals. Older adults with T2DM may face an increased risk of undernutrition due to excessively strict dietary habits aimed at managing blood sugar levels[12]. Various tools have been developed to assess malnutrition status, including the Malnutrition Screening Tool[13], Malnutrition Universal Screening Tool[14], Mini Nutritional Assessment Short Form[15], Nutrition Risk Score 2002[16], and Geriatric Nutritional Risk Index (GNRI)[17]. The GNRI has been utilized as a convenient and accessible method among these instruments for assessing outcomes, relying on serum albumin levels and the ratio of real body weight to ideal body weight.

The relationship between nutritional status and bone mass has been observed in different populations, such as individuals with chronic obstructive pulmonary disease[18], rheumatoid arthritis[19,20], and end-stage renal disease[21]. In people with T2DM, nutrition, sarcopenia, and osteoporosis are rarely explored. Therefore, in this study, we investigated associations between bone mineral density (BMD), the GNRI and body composition in patients with T2DM.

MATERIALS AND METHODS

Study design and participants

We conducted a retrospective cross-sectional study among T2DM patients admitted to the Department of Endocrinology, The Second Affiliated Hospital of Nantong University, between January 1, 2020, and March 1, 2022.

Patients

The main inclusion criterion in this study was T2DM. T2DM was defined as a fasting blood glucose level of > 7.0 mmol/L and/or a 2-h postprandial blood glucose level > 11.1 mmol/L in an oral glucose tolerance test, in accordance with the 1999 World Health Organization T2DM diagnosis and classification criteria. The patients were excluded based on the following criteria: (1) Malignant tumor and severe heart, cerebral, liver or kidney diseases; (2) pituitary, thyroid, parathyroid and adrenal diseases; (3) treatment with glucocorticoids or sex hormones in the past 6 mo; (4) concomitantly taking drugs affecting bone metabolism, such as calcium, vitamin D and bisphosphonates; and (5) unavailability of complete data on relevant variables and assessments. This study was approved by the ethics committee of The Second Affiliated Hospital of Nantong University and was in line with the Helsinki Declaration. The number for ethics approval was 2021KT063.

Data collection

Collection of demographic, medical, and laboratory data: All demographic information and relevant medical histories of the participants were recorded from their medical records. Demographic data included age, sex, height, weight and BMI. Body weight and height were measured with the patient lightly clothed and without shoes. BMI (kg/m^2) was calculated as body weight in kilograms divided by height in meters squared. Medical history included diabetes duration and history of hypertension. The duration of diabetes was calculated by months from the time that the patient was diagnosed with T2DM in their medical records to the date we took blood tests. We also collected the glucose-lowering therapy status among participants. Glucose-lowering therapies were categorized as lifestyle alone and drug therapy. Hypoglycemic agents included insulin, insulin secretagogues, insulin sensitizers, metformin, AGIs (α-glucosidase inhibitors), DPP-4Is (dipeptidyl peptidase-4 inhibitors), SGLT-2Is (sodium-glucose cotransporter-2 inhibitors) and GLP-1RAs (glucagon-like peptide-1 receptor agonists).

For laboratory data collection, the nurses in the ward took blood samples from the antecubital vein in the early morning hours after overnight fasting (at least 8 h). Triglycerides (TGs; colorimetric method), total cholesterol (TC; cholesterol oxidase method), low-density lipoprotein cholesterol (LDL-C; selective melting method) and high-density lipoprotein cholesterol (HDL-C; enzyme modification method) were measured by an automatic biochemical instrument (Model 7600, Hitachi). The level of HbA1c was assessed by ion exchange high-performance liquid chromatography. The levels of bone metabolism markers, including osteocalcin (OS), β-collagen special sequence (β-CTX) and total type I procollagen N-terminal extension peptide (TP1NP). Additionally, other biochemical markers, such as serum creatinine (Cr), uric acid (UA), albumin and total bilirubin (TBil), were measured according to standard methodology.

BMD and body composition measurements: BMD and body composition were measured using dual energy X-ray absorptiometry (DXA; Hologic-Discovery Wi, S/N86856). All of the patients were scanned, and calculations were performed by professionals in the corresponding medical and technical departments. According to the instrument manual, all operations were carried out in the standard mode: The patient lay flat and was scanned from head to feet. The measured indices included lumbar spine (L1-L4) BMD (LS-BMD), femoral neck BMD (FN-BMD), hip BMD, total (whole-body) BMD, total body fat, the android/gynoid ratio, fat mass index, lean mass index and appendicular SMI (ASMI). BMD (g/cm^2) was calculated using the following formula: Bone mineral content (g)/area (cm^2); ASMI was calculated by limb skeletal muscle mass: ASMM (kg)/height^2 (m^2); lean mass index was calculated using the following formula: Lean mass (kg)/height^2 (m^2); and fat mass index was calculated using the following formula: Fat mass (kg)/height^2 (m^2).

Calculation of the GNRI

Based on the serum albumin level and baseline body weight, the GNRI is calculated as follows: GNRI = [1.489 albumin (g/L) + (weight/ideal weight)]. Ideal weight can be further calculated by the following equations: Men: Ideal weight = height (cm) – 100 – [(height - 150)/4]; Women: Ideal weight = height (cm) – 100 – [(height - 150)/2.5].

Statistical analysis

The patients were classified by GNRI tertiles with cutoff values of < 101.85, 101.85 to 109.52, and > 109.52. A descriptive analysis of the data was performed based on the type of data, including the mean and standard deviation, and frequency and percentage. The trends of continuous data and categorical data were detected using one-way ANOVA with linear polynomial contrasts, Kruskal-Wallis tests, and Chi-squared tests with linear-by-linear associations. Furthermore, we generated scatter plots using GraphPad Prism to show the correlation between the GNRI and BMD, ASMI, and total body fat (T-FAT). The factors associated with BMD and ASMI were identified using multiple stepwise linear regression analyses.

For the statistical analysis, we employed IBM SPSS Statistics (25.0) and GraphPad Prism (9.0). Statistical significance was determined using a *P* value less than 0.05. Normally distributed values are given as the mean ± SD, skewed distributed values are given as the median (25% and 75% interquartiles), and categorical variables are given as frequency (percentage).

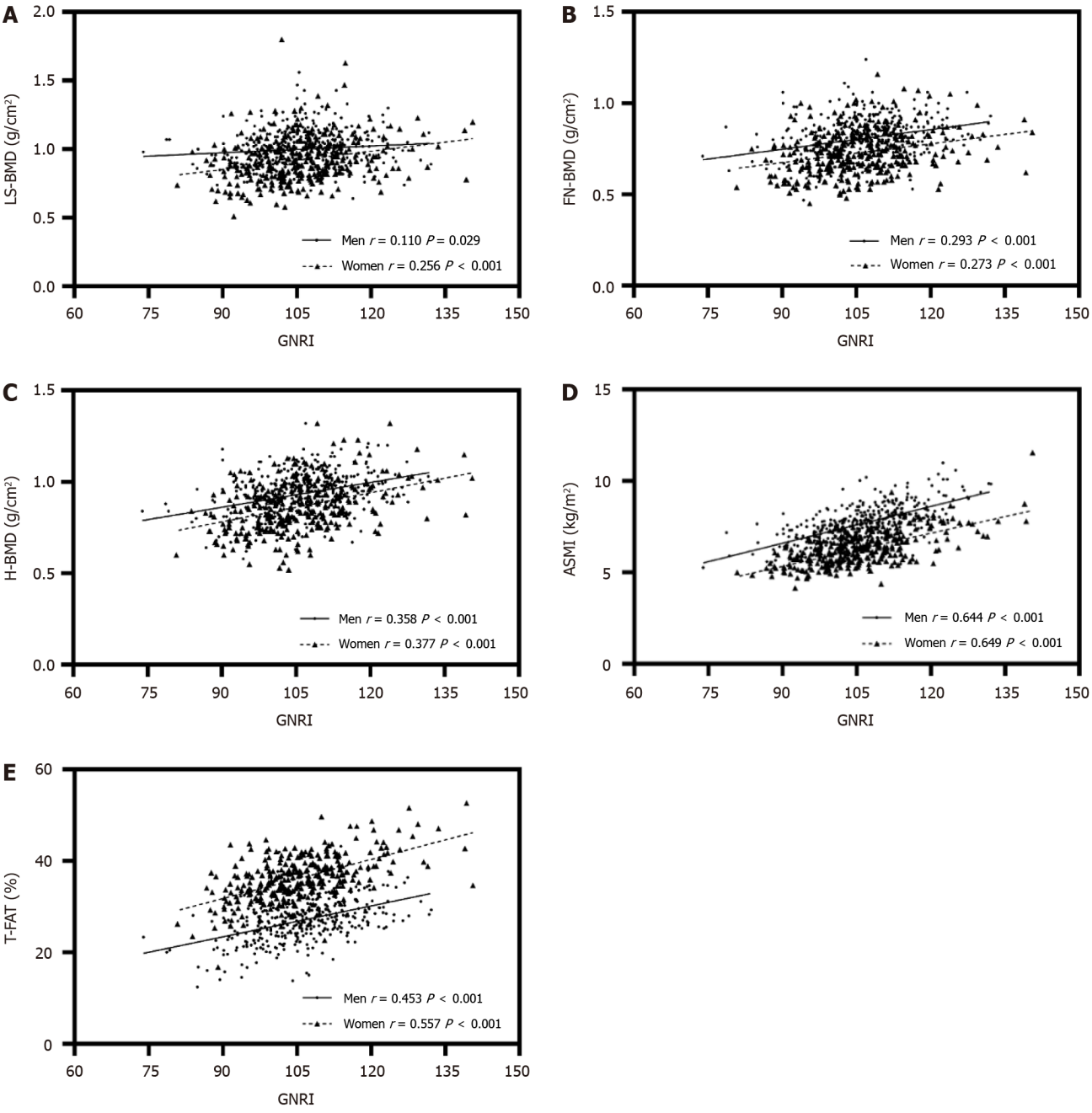

Figure 1 Scatter diagrams showing the correlation between Geriatric Nutritional Risk Index, bone mineral density, total body fat and appendicular skeletal muscle index. A: Lumbar spine (L1-L4) bone mineral density (BMD); B: Femoral neck BMD; C: Hip BMD; D: Appendicular skeletal muscle index; E: Total (whole-body) BMD. BMD: Bone mineral density; GNRI: Geriatric Nutritional Risk Index; LS-BMD: Lumbar spine (L1-L4) bone mineral density; FN-BMD: Femoral neck bone mineral density; H-BMD: Hip bone mineral density; T-FAT: Total body fat.

RESULTS

In this study, we enrolled 689 patients (57.2% men and 42.8% women), with a mean age of 55.59 ± 10.88 years.

Patient characteristics

Table 1 shows comparisons of the characteristics of the patients classified by GNRI tertiles. Compared with patients in tertile 1, those in tertile 3 tended to be younger, had lower HbA1c and β-CTX, and had higher BMI, BMD, total body fat, android/gynoid ratio, fat mass index, lean mass index, ASMI, albumin, UA, TG, TC and TBil. These important trends persisted even when the patients were divided into younger and older subgroups (Tables 2-4).

Associations between GNRI, BMD, T-FAT and ASMI

Figure 1 shows the correlation between GNRI, BMD, T-FAT and ASMI in T2DM patients; the average BMD at the lumbar spine, femur neck and total hip in men was higher than that in women (1.00 *vs* 0.92, 0.81 *vs* 0.73, 0.94 *vs* 0.86, respectively, and all $P < 0.001$); the GNRI was found to be positively and significantly associated with ASMI, T-FAT and BMD at all

Table 1 Comparison of baseline characteristics among type 2 diabetes mellitus patients stratified by tertiles of the Geriatric Nutritional Risk Index

Characteristics	Total (*n* = 689)	GNRI tertile 1 (*n* = 230)	GNRI tertile 2 (*n* = 230)	GNRI tertile 3 (*n* = 229)	*F*/*H*/χ^2	*P* value
Women [*n* (%)]	295 (42.8)	109 (47.4)	96 (41.7)	90 (39.3)	3.065	0.080
Age (yr)	55.59 ± 10.88	58.02 ± 10.49	56.00 ± 10.84	52.74 ± 10.69	28.071	< 0.001
Height (cm)	166.89 ± 8.24	165.25 ± 7.88	167.10 ± 8.52	168.33 ± 8.06	16.410	< 0.001
Weight (kg)	70.00 (62.00-80.00)	62.00 (56.00-69.00)	71.00 (64.75-78.85)	80.00 (72.75-90.00)	16.636	< 0.001
Diabetes duration (yr)	7.33 ± 6.20	8.96 ± 6.42	7.58 ± 6.44	5.45 ± 5.15	38.741	< 0.001
BMI (kg/m^2)	25.39 (23.23-27.78)	22.92 (21.31-24.49)	25.53 (24.20-26.93)	28.28 (26.62-30.47)	19.284	< 0.001
SBP (mmHg)	133.84 ± 15.34	132.96 ± 16.80	133.89 ± 15.98	134.67 ± 12.98	1.420	0.234
DBP (mmHg)	81.00 ± 9.88	79.44 ± 10.19	80.76 ± 9.50	82.79 ± 9.70	13.343	< 0.001
Hypertension [*n* (%)]	333 (48.3)	112 (48.7)	117 (50.9)	104 (45.4)	0.492	0.483
GNRI (score)	105.61 (99.64-112.01)	97.09 (93.02-99.66)	105.62 (103.73-107.68)	114.25 (112.01-119.37)	27.818	< 0.001
Glucose-lowering therapies [*n* (%)]						
Lifestyle alone	121 (17.6)	31 (13.5)	36 (15.7)	54 (23.6)	8.071	0.004
Insulin treatments	248 (36.0)	102 (44.3)	89 (38.7)	57 (24.9)	18.817	< 0.001
Insulin secretagogues	222 (32.2)	83 (36.1)	78 (33.9)	61 (26.6)	4.681	0.030
Insulin sensitizers	79 (11.5)	25 (10.9)	28 (12.2)	26 (11.4)	0.027	0.870
Metformin	322 (46.7)	88 (38.3)	110 (47.8)	124 (54.1)	11.622	< 0.001
AGIs	105 (15.2)	23 (10.0)	33 (14.3)	49 (21.4)	11.519	< 0.001
DPP-4Is	57 (8.3)	18 (7.8)	23 (10.0)	16 (7.0)	0.105	0.745
SGLT-2Is	93 (13.5)	28 (12.2)	35 (15.2)	30 (13.1)	0.085	0.771
GLP-1RAs	41 (6.0)	3 (1.3)	14 (6.1)	24 (10.5)	17.240	< 0.001
Statins	122 (17.7)	37 (16.1)	45 (19.6)	40 (17.5)	0.151	0.698
Laboratory findings						
HbA1c (%)	8.99 ± 1.85	9.50 ± 2.02	8.92 ± 1.63	8.53 ± 1.75	33.073	< 0.001
Albumin (g/L)	38.50 (36.20-41.30)	35.90 (33.88-37.63)	38.60 (37.00-40.60)	41.70 (39.45-44.00)	17.954	< 0.001
Cr (μmol/L)	58.51 ± 21.32	58.22 ± 25.22	57.85 ± 21.58	59.46 ± 16.26	0.386	0.535
UA (μmol/L)	312.68 ± 99.25	279.47 ± 103.09	314.02 ± 89.46	344.68 ± 94.19	53.222	< 0.001
TG (mmol/L)	1.89 (1.18-3.11)	1.46 (0.98-2.33)	1.81 (1.15-2.83)	2.38 (1.58-3.98)	7.626	< 0.001
TC (mmol/L)	4.41 ± 1.06	4.29 ± 1.01	4.33 ± 0.98	4.62 ± 1.15	10.890	0.001
HDL-C (mmol/L)	1.14 ± 0.27	1.15 ± 0.28	1.15 ± 0.25	1.10 ± 0.27	3.679	0.056
LDL-C (mmol/L)	2.81 ± 0.87	2.80 ± 0.88	2.80 ± 0.82	2.84 ± 0.91	0.309	0.578
TBil (μmol/L)	11.21 ± 4.71	10.25 ± 4.60	11.43 ± 4.68	11.95 ± 4.70	15.213	< 0.001
OS (ng/mL)	11.85 ± 3.99	12.06 ± 4.26	11.88 ± 3.89	11.60 ± 3.82	1.508	0.220
β-CTX (ng/mL)	0.45 ± 0.22	0.51 ± 0.25	0.44 ± 0.21	0.41 ± 0.19	25.645	< 0.001
TP1NP (ng/mL)	40.73 ± 14.53	41.00 ± 14.00	40.74 ± 14.66	40.45 ± 14.98	0.165	0.685
DXA parameters (g/cm^2)						
LS-BMD	0.97 ± 0.16	0.92 ± 0.14	0.99 ± 0.17	0.99 ± 0.15	22.118	< 0.001
FN-BMD	0.77 ± 0.12	0.73 ± 0.12	0.79 ± 0.13	0.81 ± 0.11	53.333	< 0.001
H-BMD	0.91 ± 0.13	0.85 ± 0.12	0.91 ± 0.13	0.95 ± 0.12	83.980	< 0.001

T-BMD	1.10 ± 0.12	1.07 ± 0.12	1.10 ± 0.12	1.12 ± 0.11	21.875	< 0.001
Body composition						
Total body fat (%)	31.03 ± 6.56	29.03 ± 6.55	30.80 ± 5.83	33.26 ± 6.62	51.017	< 0.001
Android/gynoid ratio	1.31 ± 0.22	1.23 ± 0.22	1.33 ± 0.21	1.36 ± 0.20	49.682	< 0.001
Fat mass index (kg/m^2)	7.53 (6.20-9.09)	6.33 (5.08-7.53)	7.54 (6.44-8.74)	8.91 (7.51-10.75)	13.010	< 0.001
Lean mass index (kg/m^2)	16.95 (15.53-18.54)	15.66 (14.44-16.83)	17.11 (15.92-18.39)	18.60 (16.99-19.90)	14.055	< 0.001
ASMI (kg/m^2)	7.09 ± 1.17	6.38 ± 0.91	7.09 ± 0.97	7.79 ± 1.1	218.066	< 0.001

BMI: Body mass index; SBP: Systolic/diastolic blood pressure; DBP: Diastolic blood pressure; GNRI: Geriatric nutritional risk index; AGIs: α-glucosidase inhibitors; DPP-4Is: Dipeptidyl peptidase-4 inhibitors; SGLT-2Is: Sodium-glucose cotransporter-2 inhibitors; GLP-1RAs: Glucagon-like peptide-1 receptor agonists; Cr: Creatinine; UA: Uric acid; TG: Triglyceride; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TBil: Total bilirubin; OS: Osteocalcin; β-CTX: β-collagen special sequence; TP1NP: Total type I procollagen N-terminal extension peptide; DXA: Dual energy X-ray absorptiometry; LS-BMD: Lumbar spine (L1-L4) bone mineral density; FN-BMD: Femoral neck bone mineral density; H-BMD: Hip bone mineral density; T-BMD: Total (whole-body) bone mineral density; ASMI: Appendicular skeletal muscle index.

bone sites in men and women; Table 5 shows multiple linear regression models displaying associations of the GNRI with BMD; the fully adjusted Model 3 further adjusted for HbA1c, OS, β-CTX, TP1NP, albumin, Cr, UA, TG, TC, HDL-C, LDL-C, TBil, and the GNRI was significantly and positively associated with LS-BMD (b = 0.040, t = 2.492, P = 0.013, R^2 = 0.197) and FN-BMD (b = 0.027, t = 2.345, P = 0.019, R^2 = 0.341).

Multivariate forward linear regression analysis of the determinants of BMD and ASMI

Table 6 shows the determinants of BMD using multivariate stepwise linear regression analysis after adjusting for age, sex, height, weight, diabetes duration, hypertension, systolic/diastolic blood pressure (SBP), diastolic blood pressure (DBP), GNRI, BMI, HbA1c, OS, β-CTX, TP1NP, albumin, Cr, UA, TG, TC, HDL-C, LDL-C, TBil, ASMI, total body fat, android/gynoid ratio, fat mass index and lean mass index; the lean mass index was positively correlated with BMD at all bone sites; age, diabetes duration and β-CTX were negatively correlated with BMD at all bone sites; height and Cr were positively correlated with lumbar spine BMD, whereas albumin and ASMI were negatively correlated with lumbar spine BMD; albumin and the android/gynoid ratio were negatively correlated with femoral neck BMD, whereas height was positively correlated with femoral neck BMD; weight was positively correlated with total hip BMD, whereas the android/gynoid ratio was negatively correlated with total hip BMD.

Table 7 shows the determinants of ASMI using multivariate forward linear regression analysis after adjusting for age, sex, height, weight, diabetes duration, hypertension, SBP, DBP, GNRI, BMI, HbA1c, OS, β-CTX, TP1NP, albumin, Cr, UA, TG, TC, HDL-C, LDL-C and TBil; in men, age, diabetes duration and HbA1c were negatively correlated with ASMI, whereas weight and BMI were positively correlated with ASMI; in women, weight and OS were positively correlated with ASMI, whereas age, height, TBil and β-CTX were negatively correlated with ASMI.

DISCUSSION

This study investigated associations among GNRI, BMD, and ASMI in T2DM patients. In this research, we discovered that proper nutrition, as denoted by a high GNRI, was linked to a lower HbA1c, higher BMD at all bone sites, higher lean mass index and higher ASMI. Based on prior research, this study utilized the GNRI and found that the GNRI was positively related to ASMI and BMD at all bone sites in T2DM patients. Additionally, a low lean mass index and higher β-CTX were associated with low BMD at all bone sites. Age was negatively correlated with ASMI, whereas weight was positively correlated with ASMI.

Despite the appropriate consumption, the nutrition of patients with T2DM was significantly impacted[22]. Diabetes speeds up the decline of muscle power, quality and serum albumin, highlighting the importance of maintaining a proper balance of protein and energy in one's diet. The current investigation demonstrated that a decreased GNRI posed a notable hazard for diminished BMD and ASMI among individuals with T2DM. This finding is consistent with previous studies[23]. Studies have demonstrated that the GNRI can be applied as a convenient and reliable indicator of the BMD and ASMI conditions of patients with chronic hepatitis C[24], postmenopausal women who have undergone total thyroidectomy[25] and patients receiving hemodialysis[26]. Therefore, the GNRI might be a convenient and reliable indicator of BMD and ASMI status in patients with T2DM. As albumin level reflects protein status and is a major component of the GNRI, the effect of protein on bone and muscle may help to explain the associations between GNRI, BMD and ASMI.

The second important finding of this study is that a low GNRI was associated with a higher HbA1c. This indicates that the presence of malnutrition is not conducive to blood sugar control. In addition to drug therapy, the basic treatment regimen for type 2 diabetes patients is diet restriction and exercise to achieve the goal of controlling blood sugar. Malnutrition can result if there is no strict and regular diet strategy. A previous study has proven that hyperglycemia contributes to the accelerated decline in muscle mass among patients with T2DM[27]. Higher HbA1c levels may lead to

Table 2 Comparison of baseline characteristics among type 2 diabetes mellitus patients stratified by the tertiles of age

Characteristics	Total (*n* = 689)	Younger[1] (*n* = 219)	Older[2] (*n* = 470)	χ^2/t/z	*P* value
Women [*n* (%)]	295 (42.8)	111 (50.7)	184 (39.1)	8.120	0.004
Age (yr)	55.59 ± 10.88	43.71 ± 7.23	61.12 ± 7.25	-29.374	< 0.001
Height (cm)	166.89 ± 8.24	167.33 ± 8.55	166.69 ± 8.1	0.951	0.342
Weight (kg)	70.00 (62.00-80.00)	71.00 (62.00-82.00)	70.00 (62.00-80.00)	-1.178	0.239
Diabetes duration (yr)	7.33 ± 6.20	4.44 ± 3.82	8.68 ± 6.62	-10.603	< 0.001
BMI (kg/m^2)	25.39 (23.23-27.78)	25.42 (23.11-28.34)	25.39 (23.32-27.55)	-1.001	0.317
SBP (mmHg)	133.84 ± 15.34	128.84 ± 13.78	136.16 ± 15.49	-5.977	< 0.001
DBP (mmHg)	81.00 ± 9.88	81.74 ± 10.15	80.65 ± 9.75	1.342	0.180
Hypertension [*n* (%)]	333 (48.3)	66 (30.1)	267 (56.8)	42.556	< 0.001
GNRI (score)	105.61 (99.64-112.01)	107.2 (101.18-113.56)	105.09 (98.93-110.98)	-2.880	0.004
Glucose-lowering therapies					
Lifestyle alone [*n* (%)]	121 (17.6)	58 (26.5)	63 (13.4)	17.653	< 0.001
Insulin treatments [*n* (%)]	248 (36.0)	73 (33.3)	175 (37.2)	0.987	0.321
Insulin secretagogues [*n* (%)]	222 (32.2)	44 (20.1)	178 (37.9)	21.627	< 0.001
Insulin sensitizers [*n* (%)]	79 (11.5)	20 (9.1)	59 (12.6)	1.722	1.189
Metformin [*n* (%)]	322 (46.7)	92 (42.0)	230 (48.9)	2.880	0.090
AGIs [*n* (%)]	105 (15.2)	29 (13.2)	76 (16.2)	0.992	0.319
DPP-4Is [*n* (%)]	57 (8.3)	19 (8.7)	38 (8.1)	0.069	0.793
SGLT-2Is [*n* (%)]	93 (13.5)	26 (11.9)	67 (14.3)	0.727	0.394
GLP-1RAs [*n* (%)]	41 (6.0)	15 (6.8)	26 (5.5)	0.463	0.496
Statins [*n* (%)]	122 (17.7)	38 (17.4)	84 (17.9)	0.028	0.868
Laboratory findings					
HbA1c (%)	8.99 ± 1.85	8.99 ± 1.9	8.98 ± 1.82	0.075	0.941
Albumin (g/L)	38.50 (36.20-41.30)	39.00 (36.8-41.6)	38.25 (35.8-41)	-2.470	0.014
Cr (μmol/L)	58.51 ± 21.32	52.09 ± 12.75	61.5 ± 23.74	-6.750	< 0.001
UA (μmol/L)	312.68 ± 99.25	317.68 ± 112.65	310.35 ± 92.39	0.903	0.367
TG (mmol/L)	1.89 (1.18-3.11)	1.99 (1.28-3.41)	1.78 (1.15-2.87)	-1.940	0.052
TC (mmol/L)	4.41 ± 1.06	4.5 ± 1.09	4.37 ± 1.04	1.475	0.141
HDL-C (mmol/L)	1.14 ± 0.27	1.09 ± 0.25	1.16 ± 0.27	-3.049	0.002
LDL-C (mmol/L)	2.81 ± 0.87	2.85 ± 0.87	2.79 ± 0.87	0.803	0.422
TBil (μmol/L)	11.21 ± 4.71	10.99 ± 4.28	11.31 ± 4.89	-0.836	0.404
OS (ng/mL)	11.85 ± 3.99	11.93 ± 3.43	11.81 ± 4.23	0.409	0.683
β-CTX (ng/mL)	0.45 ± 0.22	0.47 ± 0.21	0.45 ± 0.23	1.137	0.256
TP1NP (ng/mL)	40.73 ± 14.53	41.58 ± 13.84	40.34 ± 14.84	1.044	0.297
DXA parameters (g/cm^2)					
LS-BMD	0.97 ± 0.16	1.00 ± 0.14	0.95 ± 0.16	4.126	< 0.001
FN-BMD	0.77 ± 0.12	0.82 ± 0.12	0.75 ± 0.12	6.360	< 0.001
H-BMD	0.91 ± 0.13	0.94 ± 0.12	0.89 ± 0.13	5.441	< 0.001
T-BMD	1.10 ± 0.12	1.13 ± 0.1	1.08 ± 0.12	4.643	< 0.001
Body composition					
Total body fat (%)	31.03 ± 6.56	31.76 ± 6.25	30.69 ± 6.68	2.001	0.046

Android/gynoid ratio	1.31 ± 0.22	1.3 ± 0.22	1.31 ± 0.21	-0.107	0.915
Fat mass index (kg/m^2)	7.53 (6.20-9.09)	7.94 (6.55-9.26)	7.32 (6.17-8.99)	-2.621	0.009
Lean mass index (kg/m^2)	16.95 (15.53-18.54)	17.01 (15.45-18.66)	16.94 (15.56-18.51)	-0.952	0.341
ASMI (kg/m^2)	7.09 ± 1.17	7.23 ± 1.31	7.02 ± 1.09	2.026	0.043

[1]Men aged < 50 years and women aged < 55 years.
[2]Men aged ≥ 50 years and postmenopausal women aged ≥ 55 years.
BMI: Body mass index; SBP: Systolic/diastolic blood pressure; DBP: Diastolic blood pressure; GNRI: Geriatric nutritional risk index; AGIs: α-glucosidase inhibitors; DPP-4Is: Dipeptidyl peptidase-4 inhibitors; SGLT-2Is: Sodium-glucose cotransporter-2 inhibitors; GLP-1RAs: Glucagon-like peptide-1 receptor agonists; Cr: Creatinine; UA: Uric acid; TG: Triglyceride; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TBil: Total bilirubin; OS: Osteocalcin; β-CTX: β-collagen special sequence; TP1NP: Total type I procollagen N-terminal extension peptide; DXA: Dual energy X-ray absorptiometry; LS-BMD: Lumbar spine (L1-L4) bone mineral density; FN-BMD: Femoral neck bone mineral density; H-BMD: Hip bone mineral density; T-BMD: Total (whole-body) bone mineral density; ASMI: Appendicular skeletal muscle index.

Table 3 Comparison of baseline characteristics among younger type 2 diabetes mellitus patients

Characteristics	Total (*n* = 219)	GNRI tertile 1 (*n* = 63)	GNRI tertile 2 (*n* = 68)	GNRI tertile 3 (*n* = 88)	*F*/*H*/χ^2	*P* value
Women [*n* (%)]	111 (50.7)	40 (63.5)	33 (48.5)	38 (43.5)	5.786	0.016
Age (yr)	43.71 ± 7.23	45.87 ± 6.84	43.04 ± 6.45	42.67 ± 7.80	6.880	0.009
Height (cm)	167.33 ± 8.55	164.57 ± 8.13	167.1 ± 8.49	169.48 ± 8.38	12.759	< 0.001
Weight (kg)	71.00 (62.00-82.00)	60.00 (56.00-66.70)	70.00 (64.00-76.85)	83.05 (74.25-93.20)	10.568	< 0.001
Diabetes duration (yr)	4.44 ± 3.82	5.84 ± 3.91	4.82 ± 4.03	3.15 ± 3.14	20.524	< 0.001
BMI (kg/m^2)	25.42 (23.11-28.34)	22.72 (21.10-23.88)	25.32 (23.84-26.57)	28.60 (26.97-31.73)	11.641	< 0.001
SBP (mmHg)	128.84 ± 13.78	126.62 ± 13.78	123.6 ± 13.08	134.49 ± 12.31	16.237	< 0.001
DBP (mmHg)	81.74 ± 10.15	80.22 ± 10.41	77.99 ± 7.75	85.72 ± 10.30	14.496	< 0.001
Hypertension [*n* (%)]	66 (30.1)	17 (27.0)	21 (30.9)	28 (31.8)	0.383	0.536
GNRI (score)	107.2 (101.18-113.56)	97.28 (92.85-99.92)	105.69 (104.20-107.71)	114.90 (112.15-120.21)	15.551	< 0.001
Glucose-lowering therapies [*n* (%)]						
Lifestyle alone	58 (26.5)	14 (22.2)	12 (17.6)	32 (36.4)	4.469	0.035
Insulin treatments	73 (33.3)	28 (44.4)	30 (44.1)	15 (17.0)	13.761	< 0.001
Insulin secretagogues	44 (20.1)	12 (19.0)	18 (26.5)	14 (15.9)	0.382	0.536
Insulin sensitizers	20 (9.1)	6 (9.5)	6 (8.8)	8 (9.1)	0.006	0.936
Metformin	92 (42.0)	20 (31.7)	31 (45.6)	41 (46.6)	2.617	0.106
AGIs	29 (13.2)	6 (9.5)	7 (10.3)	16 (18.2)	11.519	< 0.001
DPP-4Is	19 (8.7)	4 (6.3)	9 (13.2)	6 (6.8)	0.002	0.961
SGLT-2Is	26 (11.9)	11 (17.5)	8 (11.8)	7 (8.0)	3.118	0.077
GLP-1RAs	15 (6.8)	0 (0.0)	5 (7.4)	10 (11.4)	7.234	0.007
Statins	38 (17.4)	12 (19.0)	12 (17.6)	14 (15.9)	0.256	0.613
Laboratory findings						
HbA1c (%)	8.99 ± 1.9	9.58 ± 2.26	9.07 ± 1.66	8.52 ± 1.69	12.151	0.001
Albumin (g/L)	39.00 (36.8-41.6)	36.20 (34.60-37.90)	39.15 (37.20-40.75)	41.60 (39.00-44.23)	9.497	< 0.001
Cr (μmol/L)	52.09 ± 12.75	48.4 ± 13.54	51.31 ± 10.27	55.35 ± 13.2	11.693	0.001
UA (μmol/L)	317.68 ± 112.65	270.22 ± 140.24	313.84 ± 90.09	354.62 ± 92.24	22.682	< 0.001
TG (mmol/L)	1.99 (1.28-3.41)	1.50 (0.98-2.02)	2.03 (1.12-3.52)	2.46 (1.84-4.42)	5.412	< 0.001

TC (mmol/L)	4.5 ± 1.09	4.37 ± 0.94	4.3 ± 0.85	4.75 ± 1.30	5.389	0.021
HDL-C (mmol/L)	1.09 ± 0.25	1.15 ± 0.26	1.08 ± 0.21	1.06 ± 0.26	4.644	0.032
LDL-C (mmol/L)	2.85 ± 0.87	2.92 ± 0.91	2.86 ± 0.79	2.79 ± 0.92	0.850	0.358
TBil (μmol/L)	10.99 ± 4.28	9.78 ± 3.67	11.11 ± 4.15	11.76 ± 4.63	7.855	0.006
OS (ng/mL)	11.93 ± 3.43	12.05 ± 3.14	11.77 ± 3.07	11.98 ± 3.90	0.008	0.931
β-CTX (ng/mL)	0.47 ± 0.21	0.53 ± 0.24	0.45 ± 0.19	0.44 ± 0.20	5.436	0.021
TP1NP (ng/mL)	41.58 ± 13.84	41.95 ± 11.58	40.21 ± 13.02	42.37 ± 15.87	0.075	0.784
DXA parameters (g/cm^2)						
LS-BMD	1.00 ± 0.14	0.96 ± 0.14	1.01 ± 0.13	1.02 ± 0.15	7.426	0.007
FN-BMD	0.82 ± 0.12	0.77 ± 0.11	0.82 ± 0.12	0.85 ± 0.11	18.433	< 0.001
H-BMD	0.94 ± 0.12	0.88 ± 0.11	0.94 ± 0.11	0.99 ± 0.11	34.357	< 0.001
T-BMD	1.13 ± 0.1	1.1 ± 0.11	1.12 ± 0.09	1.15 ± 0.10	8.681	0.004
Body composition						
Total body fat (%)	31.76 ± 6.25	29.85 ± 5.69	31.24 ± 5.90	33.53 ± 6.49	13.922	< 0.001
Android/gynoid ratio	1.3 ± 0.22	1.21 ± 0.22	1.31 ± 0.19	1.37 ± 0.21	22.631	< 0.001
Fat mass index (kg/m^2)	7.94 (6.55-9.26)	6.56 (5.61-7.63)	7.89 (6.52-8.88)	9.13 (7.71-11.21)	7.905	< 0.001
Lean mass index (kg/m^2)	17.01 (15.45-18.66)	15.25 (14.14-16.62)	16.93 (15.82-18.37)	18.65 (17.26-20.34)	9.380	< 0.001
ASMI (kg/m^2)	7.23 ± 1.31	6.23 ± 0.90	7.09 ± 1.00	8.04 ± 1.24	105.442	< 0.001

BMI: Body mass index; SBP: Systolic/diastolic blood pressure; DBP: Diastolic blood pressure; GNRI: Geriatric nutritional risk index; AGIs: α-glucosidase inhibitors; DPP-4Is: Dipeptidyl peptidase-4 inhibitors; SGLT-2Is: Sodium-glucose cotransporter-2 inhibitors; GLP-1RAs: Glucagon-like peptide-1 receptor agonists; Cr: Creatinine; UA: Uric acid; TG: Triglyceride; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TBil: Total bilirubin; OS: Osteocalcin; β-CTX: β-collagen special sequence; TP1NP: Total type I procollagen N-terminal extension peptide; DXA: Dual energy X-ray absorptiometry; LS-BMD: Lumbar spine (L1-L4) bone mineral density; FN-BMD: Femoral neck bone mineral density; H-BMD: Hip bone mineral density; T-BMD: Total (whole-body) bone mineral density; ASMI: Appendicular skeletal muscle index.

Table 4 Comparison of baseline characteristics among older type 2 diabetes mellitus patients

Characteristics	Total (*n* = 470)	GNRI tertile 1 (*n* = 167)	GNRI tertile 2 (*n* = 162)	GNRI tertile 3 (*n* = 141)	*F*/*H*/χ^2	*P* value
Women [*n* (%)]	184 (39.1)	69 (41.3)	63 (38.9)	52 (36.9)	0.636	0.425
Age (yr)	61.12 ± 7.25	62.6 ± 7.56	61.43 ± 7.01	59.02 ± 6.70	18.962	< 0.001
Height (cm)	166.69 ± 8.1	165.5 ± 7.80	167.1 ± 8.55	167.62 ± 7.79	5.425	0.020
Weight (kg)	70.00 (62.00-80.00)	62.00 (57.00-70.00)	72.50 (65.00-80.00)	80.00 (72.00-85.50)	12.768	< 0.001
Diabetes duration (yr)	8.68 ± 6.62	10.13 ± 6.79	8.74 ± 6.90	6.89 ± 5.62	18.945	< 0.001
BMI (kg/m^2)	25.39 (23.32-27.55)	23.05 (21.34-24.57)	25.71 (24.33-27.11)	28.01 (26.51-29.75)	15.296	< 0.001
SBP (mmHg)	136.16 ± 15.49	135.35 ± 17.25	138.2 ± 15.13	134.78 ± 13.42	0.049	0.825
DBP (mmHg)	80.65 ± 9.75	79.15 ± 10.12	81.93 ± 9.93	80.96 ± 8.86	2.998	0.084
Hypertension [*n* (%)]	267 (56.8)	95 (56.9)	96 (59.3)	76 (53.9)	0.237	0.626
GNRI (score)	105.09 (98.93-110.98)	97.09 (93.08-99.55)	105.57 (103.59-107.63)	113.94 (111.77-118.57)	22.930	< 0.001
Glucose-lowering therapies [*n* (%)]						
Lifestyle alone	63 (13.4)	17 (10.2)	24 (14.8)	22 (15.6)	2.019	0.155
Insulin treatments	175 (37.2)	74 (44.3)	59 (36.4)	42 (29.8)	6.938	0.008
Insulin secretagogues	178 (37.9)	71 (42.5)	60 (37.0)	47 (33.3)	2.771	0.096
Insulin sensitizers	59 (12.6)	19 (11.4)	22 (13.6)	18 (12.8)	0.152	0.697

Metformin	230 (48.9)	68 (40.7)	79 (48.8)	83 (58.9)	10.012	0.002
AGIs	76 (16.2)	17 (10.2)	26 (16.0)	33 (23.4)	9.802	0.002
DPP-4Is	38 (8.1)	14 (8.4)	14 (8.6)	10 (7.1)	0.158	0.691
SGLT-2Is	67 (14.3)	17 (10.2)	27 (16.7)	23 (16.3)	2.509	0.113
GLP-1RAs	26 (5.5)	3 (1.8)	9 (5.6)	14 (9.9)	9.636	0.002
Statins	84 (17.9)	25 (15.0)	33 (20.4)	26 (18.4)	0.707	0.400
Laboratory findings						
HbA1c (%)	8.98 ± 1.82	9.47 ± 1.93	8.86 ± 1.61	8.54 ± 1.79	21.198	< 0.001
Albumin (g/L)	38.25 (35.8-41)	35.80 (33.50-37.30)	38.40 (36.70-40.60)	41.70 (39.70-44.00)	15.071	< 0.001
Cr (μmol/L)	61.5 ± 23.74	61.93 ± 27.54	60.59 ± 24.35	62.03 ± 17.46	0.000	0.994
UA (μmol/L)	310.35 ± 92.39	282.96 ± 85.27	314.1 ± 89.47	338.47 ± 95.18	29.509	< 0.001
TG (mmol/L)	1.78 (1.15-2.87)	1.42 (0.97-2.44)	1.75 (1.18-2.62)	2.29 (1.41-3.83)	5.347	< 0.001
TC (mmol/L)	4.37 ± 1.04	4.27 ± 1.04	4.34 ± 1.03	4.53 ± 1.04	4.991	0.026
HDL-C (mmol/L)	1.16 ± 0.27	1.15 ± 0.29	1.18 ± 0.26	1.13 ± 0.27	0.348	0.555
LDL-C (mmol/L)	2.79 ± 0.87	2.75 ± 0.87	2.77 ± 0.84	2.87 ± 0.9	1.525	0.218
TBil (μmol/L)	11.31 ± 4.89	10.43 ± 4.91	11.56 ± 4.89	12.06 ± 4.75	8.890	0.003
OS (ng/mL)	11.81 ± 4.23	12.07 ± 4.63	11.93 ± 4.19	11.37 ± 3.76	1.992	0.159
β-CTX (ng/mL)	0.45 ± 0.23	0.5 ± 0.25	0.44 ± 0.22	0.39 ± 0.18	22.076	< 0.001
TP1NP (ng/mL)	40.34 ± 14.84	40.65 ± 14.83	40.96 ± 15.33	39.26 ± 14.33	0.617	0.433
DXA parameters (g/cm^2)						
LS-BMD	0.95 ± 0.16	0.91 ± 0.15	0.98 ± 0.18	0.97 ± 0.14	12.015	0.001
FN-BMD	0.75 ± 0.12	0.71 ± 0.11	0.77 ± 0.13	0.78 ± 0.10	29.138	< 0.001
H-BMD	0.89 ± 0.13	0.84 ± 0.12	0.9 ± 0.14	0.93 ± 0.11	45.242	< 0.001
T-BMD	1.08 ± 0.12	1.06 ± 0.12	1.09 ± 0.13	1.1 ± 0.11	10.818	0.001
Body composition						
Total body fat (%)	30.69 ± 6.68	28.72 ± 6.83	30.62 ± 5.81	33.09 ± 6.71	34.740	< 0.001
Android/gynoid ratio	1.31 ± 0.21	1.24 ± 0.22	1.33 ± 0.21	1.36 ± 0.19	28.386	< 0.001
Fat mass index (kg/m^2)	7.32 (6.17-8.99)	6.26 (4.97-7.51)	7.38 (6.42-8.59)	8.70 (7.20-10.56)	10.212	< 0.001
Lean mass index (kg/m^2)	16.94 (15.56-18.51)	15.82 (14.71-17.03)	17.17 (15.92-18.43)	18.56 (16.77-19.81)	10.488	< 0.001
ASMI (kg/m^2)	7.02 ± 1.09	6.44 ± 0.91	7.09 ± 0.97	7.63 ± 1.08	112.733	< 0.001

BMI: Body mass index; SBP: Systolic/diastolic blood pressure; DBP: Diastolic blood pressure; GNRI: Geriatric nutritional risk index; AGIs: α-glucosidase inhibitors; DPP-4Is: Dipeptidyl peptidase-4 inhibitors; SGLT-2Is: Sodium-glucose cotransporter-2 inhibitors; GLP-1RAs: Glucagon-like peptide-1 receptor agonists; Cr: Creatinine; UA: Uric acid; TG: Triglyceride; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TBil: Total bilirubin; OS: Osteocalcin; β-CTX: β-collagen special sequence; TP1NP: Total type I procollagen N-terminal extension peptide; DXA: Dual energy X-ray absorptiometry; LS-BMD: Lumbar spine (L1-L4) bone mineral density; FN-BMD: Femoral neck bone mineral density; H-BMD: Hip bone mineral density; T-BMD: Total (whole-body) bone mineral density; ASMI: Appendicular skeletal muscle index.

Table 5 Multiple linear regression models displaying associations of the Geriatric Nutritional Risk Index with bone mineral density

Models	B (95%CI)	*β*	*t* value	*P* value	Adjusted R^2 for model
Lumbar spine BMD					
Model 0[1]	0.003 (0.002 to 0.004)	0.186	4.952	< 0.001	0.033
Model 1[2]	-0.002 (-0.004 to 0.000)	-0.113	-1.919	0.055	0.150
Model 2[3]	-0.002 (-0.004 to 0.000)	-0.105	-1.765	0.078	0.153

Model 3[4]	0.040 (0.008 to 0.071)	2.402	2.492	0.013	0.197
Femoral neck BMD					
Model 0[1]	0.004 (0.003 to 0.005)	0.281	7.664	< 0.001	0.077
Model 1[2]	-0.001 (-0.002 to 0.000)	-0.071	-1.32	0.187	0.293
Model 2[3]	-0.001 (-0.002 to 0.000)	-0.06	-1.12	0.263	0.306
Model 3[4]	0.027 (0.004 to 0.049)	2.047	2.345	0.019	0.341
Total hip BMD					
Model 0[1]	0.005 (0.004 to 0.006)	0.363	10.213	< 0.001	0.131
Model 1[2]	0.000 (-0.002 to 0.001)	-0.034	-0.636	0.525	0.312
Model 2[3]	0.000 (-0.002 to 0.001)	-0.025	-0.477	0.634	0.318
Model 3[4]	0.021 (-0.003 to 0.044)	1.52	1.745	0.082	0.343

[1]Unadjusted model.
[2]Adjusted for age, sex, diabetes duration, hypertension, SBP, DBP, and BMI.
[3]Additionally adjusted for antidiabetic treatments and statin medications.
[4]Additionally adjusted for HbA1c, Osteocalcin, β-collagen special sequence, total type I procollagen N-terminal extension peptide, albumin, Creatinine, uric acid, triglyceride, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, total bilirubin.
95%CI: 95% confidence interval; BMD: Bone mineral density.

Table 6 Determinants of bone mineral density using multivariate forward linear regression analysis

Factors	B	SE	*t* value	*P* value	95%CI	
					Lower	Upper
Lumbar spine BMD						
Age	-0.002	0.001	-3.754	< 0.001	-0.003	-0.001
Height	0.003	0.001	3.669	< 0.001	0.001	0.005
Diabetes duration	-0.003	0.001	-2.665	0.008	-0.005	-0.001
Albumin	-0.004	0.001	-2.864	0.004	-0.007	-0.001
Cr	0.001	0.000	1.983	0.048	0.000	0.001
β-CTX	-0.089	0.033	-2.724	0.007	-0.153	-0.025
TP1NP	-0.001	0.000	-1.633	0.103	-0.002	0.000
ASMI	-0.040	0.016	-2.449	0.015	-0.072	-0.008
Lean mass index	0.036	0.008	4.498	< 0.001	0.020	0.051
Femoral neck BMD						
Age	-0.003	0	-6.8	< 0.001	-0.003	-0.002
Height	0.003	0.001	4.672	< 0.001	0.002	0.004
Diabetes duration	-0.002	0.001	-3.617	< 0.001	-0.004	-0.001
Albumin	-0.003	0.001	-2.698	0.007	-0.005	-0.001
β-CTX	-0.100	0.018	-5.589	< 0.001	-0.135	-0.065
Android/gynoid ratio	-0.045	0.02	-2.259	0.024	-0.084	-0.006
ASMI	0.011	0.012	0.963	0.336	-0.012	0.034
Lean mass index	0.014	0.006	2.457	0.014	0.003	0.025
Total hip BMD						
Age	-0.002	0.000	-5.451	< 0.001	-0.003	-0.001
Weight	0.002	0.001	3.142	0.002	0.001	0.003
Diabetes duration	-0.002	0.001	-3.327	0.001	-0.004	-0.001

β-CTX	-0.082	0.019	-4.403	< 0.001	-0.118	-0.045
Android/gynoid ratio	-0.041	0.02	-2.003	0.046	-0.081	-0.001
Lean mass index	0.020	0.003	6.266	< 0.001	0.014	0.026

95%CI: 95% confidence interval; BMD: Bone mineral density; Cr: Creatinine; β-CTX: β-collagen special sequence; TP1NP: Total type I procollagen N-terminal extension peptide; ASMI: Appendicular skeletal muscle index.

Table 7 Determinants of appendicular skeletal muscle index using multivariate forward linear regression analysis

Factors	B	SE	*t* value	*P* value	95%CI	
					Lower	Upper
Men						
Age	-0.012	0.003	-3.721	< 0.001	-0.019	-0.006
Weight	0.019	0.006	2.983	0.003	0.006	0.031
Diabetes duration	-0.014	0.005	-2.587	0.010	-0.025	-0.003
BMI	0.136	0.022	6.238	< 0.001	0.093	0.178
HbA1c	-0.057	0.017	-3.449	0.001	-0.090	-0.025
Women						
Age	-0.008	0.003	-2.811	0.005	-0.014	-0.003
Weight	0.066	0.003	20.846	< 0.001	0.059	0.072
Height	-0.030	0.007	-4.491	< 0.001	-0.043	-0.017
TBil	-0.017	0.007	-2.369	0.018	-0.032	-0.003
OS	0.019	0.009	2.147	0.033	0.002	0.037
β-CTX	-0.536	0.175	-3.059	0.002	-0.881	-0.191

95%CI: 95% confidence interval; BMI: Body mass index; TBil: Total bilirubin; OS: Osteocalcin; β-CTX: β-collagen special sequence.

an increased risk of low muscle mass *via* a variety of mechanisms. The main causes include insulin resistance, inflammation, and the production of glycation end products. Therefore, nutritional balance is beneficial to control blood sugar and reduce the incidence of sarcopenia. Individuals with type 2 diabetes, especially the elderly, need individualized dietary strategies to reduce the incidence of malnutrition. Regular nutritional assessments are necessary. People with type 2 diabetes can avoid the adverse effects of malnutrition by adjusting their diet.

At all bone sites, there was a correlation between low BMD and a high level of β-CTX, which is the third significant discovery of this research. β-CTX is derived from the degradation of type I collagen, and its content in bone collagen is much higher than that in the rest of the tissue, so it can be more representative and more directly reflect the degradation of bone matrix collagen and be used as an indicator of bone resorption. Bone homeostasis depends on the resorption and formation of bones. Long-term hyperglycemia can affect the adhesion of osteoblasts to collagen, causing dysfunction of osteoblasts, inhibiting bone formation and accelerating bone resorption, causing an increase in PINP and β-CTX. This may explain our finding of an association between a high β-CTX level and low BMD. β-CTX plays a critical role in bone turnover and is a sensitive marker for the early diagnosis of osteoporosis.

Another important finding of this study is that age was negatively correlated with ASMI. Sarcopenia is the age-related loss of muscle mass, strength, and function[28]. Degenerative changes in the structure and function of the human neuromuscular system occur with age, and the presence of diabetes accelerates the decline in muscle mass and strength through changes such as high levels of reactive oxygen species produced by oxidative stress and dysfunctional mitochondria. In this study, we also found a significant association between weight and ASMI. The majority of studies have shown that low BMI is also associated with sarcopenia[29]. Malnutrition, a potent risk factor for sarcopenia, could potentially account for the higher occurrence and frequency of sarcopenia in individuals with reduced body weight. Malnutrition, a potent risk factor for sarcopenia, might well explain the increased prevalence and incidence of sarcopenia in individuals with lower weight.

This study had multiple limitations. Because the study had a cross-sectional design, it was not possible to establish causal relationships. Furthermore, the participants chosen for this research encompassed both males and females spanning a wide age bracket of 21 to 81 years. T2DM patients of the same gender and age range have not been studied, but this study is closer to the clinical situation. Also, we only included participants who were hospitalized; we did not evaluate muscle strength and quality. In the end, although we did not consider that environmental pollutants (mainly air

pollutants) is able to significantly affect both the clinical features of T2DM (mainly onset of disease and blood sugar control) and the nutritional status, we selected participants who lived in the same area for a long time. In the future, we will consider selecting participants from different regions of China for further research.

CONCLUSION

Poor nutrition, as indicated by a low GNRI, was associated with low levels of ASMI and BMD at all bone sites in T2DM patients. Using the GNRI to evaluate nutritional status and using DXA to investigate body composition in patients with T2DM is of value in assessing bone health and physical performance.

ARTICLE HIGHLIGHTS

Research background

In people with type 2 diabetes mellitus (T2DM), the association between nutrition, sarcopenia, and osteoporosis has rarely been explored.

Research motivation

The relationship between nutritional status and bone mass has been observed in different populations, including individuals with chronic obstructive pulmonary disease, rheumatoid arthritis, and end-stage renal disease.

Research objectives

Assess the associations among nutrition, bone mineral density (BMD) and body composition in patients with T2DM.

Research methods

A total of 689 patients with T2DM were included to perform a retrospective analysis. The general information and biochemical indices of these patients were statistically analyzed.

Research results

Those who had a high Geriatric Nutritional Risk Index (GNRI) tended to be younger and had lower HbA1c, higher BMD at all bone sites, and higher appendicular skeletal muscle index.

Research conclusions

Poor nutrition, as indicated by a low GNRI, was associated with low levels of ASMI and BMD at all bone sites in type 2 diabetes mellitus patients.

Research perspectives

We used a retrospective study to explore the association between nutrition, sarcopenia, and osteoporosis in patients with T2DM.

FOOTNOTES

Author contributions: Zhu XX and Wang LH designed the research; Zhu XX and Yao KF collected the data; Zhu XX and Huang HY analyzed the data; Zhu XX and Yao KF wrote the paper; Wang LH reviewed the paper.

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ORIGINAL ARTICLE

Case Control Study

Predictive value of angiopoietin-like protein 8 in metabolic dysfunction-associated fatty liver disease and its progression: A case-control study

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Abstract

BACKGROUND

The prevalence of metabolic dysfunction-associated fatty liver disease (MAFLD) is rapidly increasing, currently affecting approximately 25% of the global population. Liver fibrosis represents a crucial stage in the development of MAFLD, with advanced liver fibrosis elevating the risks of cirrhosis and hepatocellular carcinoma. Simple serum markers are less effective in diagnosing liver fibrosis compared to more complex markers. However, imaging techniques like transient elastography face limitations in clinical application due to equipment and technical constraints. Consequently, it is imperative to identify a straightforward yet effective method for assessing MAFLD-associated liver fibrosis.

AIM

To investigate the predictive value of angiopoietin-like protein 8 (ANGPTL8) in MAFLD and its progression.

METHODS

We analyzed 160 patients who underwent abdominal ultrasonography in the Endocrinology Department, Xiaogan Central Hospital affiliated to Wuhan University of Science and Technology, during September 2021-July 2022. Using abdominal ultrasonography and MAFLD diagnostic criteria, among the 160

patients, 80 patients (50%) were diagnosed with MAFLD. The MAFLD group was divided into the liver fibrosis group (n = 23) and non-liver fibrosis group (n = 57) by using a cut-off fibrosis-4 index ≥ 1.45. Logistical regression was used to analyze the risk of MAFLD and the risk factors for its progression. Receiver operating characteristic curves were used to evaluate the predictive value of serum ANGPTL8 in MAFLD and its progression.

RESULTS

Compared with non-MAFLD patients, MAFLD patients had higher serum ANGPTL8 and triglyceride-glucose (TyG) index (both P < 0.05). Serum ANGPTL8 (r = 0.576, P < 0.001) and TyG index (r = 0.473, P < 0.001) were positively correlated with MAFLD. Serum ANGPTL8 was a risk factor for MAFLD [odds ratio (OR): 1.123, 95% confidence interval (CI): 1.066-1.184, P < 0.001). Serum ANGPTL8 and ANGPTL8 + TyG index predicted MAFLD [area under the curve (AUC): 0.832 and 0.886, respectively; both P < 0.05]. Compared with MAFLD patients without fibrosis, those with fibrosis had higher serum ANGPTL8 and TyG index (both P < 0.05), and both parameters were positively correlated with MAFLD-associated fibrosis. Elevated serum ANGPTL8 (OR: 1.093, 95%CI: 1.044-1.144, P < 0.001) and TyG index (OR: 2.383, 95%CI: 1.199-4.736, P < 0.013) were risk factors for MAFLD-associated fibrosis. Serum ANGPTL8 and ANGPTL8 + TyG index predicted MAFLD-associated fibrosis (AUC: 0.812 and 0.835, respectively; both P < 0.05).

CONCLUSION

The serum levels of ANGPTL8 are elevated and positively correlated with MAFLD. They can serve as predictors for the risk of MAFLD and liver fibrosis, with the ANGPTL8 + TyG index potentially exhibiting even higher predictive value.

Key Words: Angiopoietin-like protein 8; Metabolic dysfunction-associated fatty liver disease; Fibrosis-4 index; Liver fibrosis

Core Tip: This study unveils elevated serum levels of angiopoietin-like protein 8 (ANGPTL8) in individuals with metabolic dysfunction-associated fatty liver disease (MAFLD). Serum ANGPTL8 emerges not only as a predictive factor for MAFLD risk but also as a powerful indicator for the presence of liver fibrosis in MAFLD. The amalgamation of serum ANGPTL8 with the triglyceride-glucose index demonstrates potential for heightened predictive accuracy in both conditions. Further exploration of serum ANGPTL8 holds promise for enhancing clinical strategies in the prevention and treatment of MAFLD.

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INTRODUCTION

Metabolic dysfunction-associated fatty liver disease (MAFLD), formerly known as non-alcoholic fatty liver disease (NAFLD), is a collective term that encompasses a range of liver diseases from simple steatosis, non-alcoholic steatohepatitis, and liver fibrosis to MAFLD-related cirrhosis and hepatocellular carcinoma[1]. All histological stages of MAFLD are significantly associated with increased overall mortality, and as the disease progresses, the overall mortality increases [2]. The National Health and Nutrition Examination Survey also reported that MAFLD and its associated metabolic disorders are significantly associated with overall and cardiovascular mortality; in addition, the survey found a significant upward trend in the prevalence of MAFLD, which has increased by 14% in the past 3 decades[3]. Nevertheless, there is still a lack of simple and effective methods for the diagnosis of this disease, so it is crucial to find a convenient laboratory index for the early identification of MAFLD and for monitoring its progression.

Angiopoietin-like protein 8 (ANGPTL8) is a 22-kDa protein composed of 198 amino acids; the ANGPTL8 gene is located on chromosome 19p13.2. ANGPTL8 is mainly expressed in the liver, white adipose tissue, and brown adipose tissue, and plays an important role in lipid flux, glucose regulation, and chronic inflammation[4,5]. Many studies have shown that the ANGPTL8 concentration is significantly higher in patients with metabolic diseases such as diabetes[6], obesity[7], and metabolic syndrome[8] than in healthy individuals. Since the pathogenesis of MAFLD is driven by metabolic disorders and changes in glucose-insulin homeostasis, we speculated that ANGPTL8 may be associated with MAFLD. To validate this hypothesis and explore the relationship between MAFLD and ANGPTL8, we evaluated the value of ANGPTL8 in predicting MAFLD and its progression. We hope to provide a simple reference index for the early prediction and prompt management of MAFLD in clinical practice.

MATERIALS AND METHODS

Study subjects

We collected the clinical data of 160 patients who underwent abdominal ultrasound examination and were admitted to the Endocrinology Department of Xiaogan Central Hospital, Wuhan University of Science and Technology, between September 2021 and July 2022. The following exclusion criteria were applied: (1) Age < 18 years or > 85 years; (2) patients with mental illness or malignant tumor; (3) patients with autoimmune diseases, active viral hepatitis, drug-induced liver damage, genetic diseases, or chronic schistosomiasis; and (4) patients with acute infection or severe cardiopulmonary, kidney, or cerebrovascular diseases or malnutrition. A total of 160 patients were enrolled according to the above criteria. MAFLD was diagnosed using a combination of abdominal ultrasound findings and relevant diagnostic criteria from the International Expert Consensus on MAFLD[1]: Evidence of fat accumulation in the liver combined with one of the following three criteria, namely, overweight/obesity, presence of type 2 diabetes mellitus, or evidence of metabolic dysregulation (type 2 diabetes was diagnosed using the 1999 World Health Organization diabetes diagnostic criteria). Patients with MAFLD were further divided into those with hepatic fibrosis [defined as fibrosis-4 (FIB-4) index ≥ 1.45] and those without hepatic fibrosis (FIB-4 index < 1.45). The study protocol was approved by the ethics committee of Xiaogan Central Hospital, and all study subjects signed informed consent forms.

Data collection

The age, gender, medical history, height, weight, waist circumference, hip circumference, and blood pressure of all patients were obtained from their medical records. The levels of the following indicators were collected through the Hospital Information Management System: Platelet count (PLT), alanine transaminase (ALT), aspartate transaminase (AST), triglyceride (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), glycated hemoglobin (HbA1c), fasting plasma glucose (FPG), and C-reactive protein (CRP). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: HOMA-IR = [FPG (mmol/L) × fasting insulin (FINS) (μU/mL)]/22.5, where FINS stands for fasting insulin level. The homeostasis model assessment of β-cell function (HOMA-β) was calculated using the following formula: HOMA-β = [20 × FINS (μU/mL)]/[FPG (mmol/L) - 3.5]. The TG-glucose (TyG) index was calculated using the formula: TyG = Ln [TG (mg/dL) × FPG (mg/mL)/2]. Liver fibrosis was evaluated using the non-invasive FIB-4 index, which was calculated as follows: FIB-4 = [age (years) × AST (U/L)]/[PLT (× 10^9/L) × $ALT^{1/2}$ (U/L)]. FIB-4 index ≥ 1.45 indicated the presence of liver fibrosis[9, 10].

ANGPTL8 detection

Serum ANGPTL8 concentration was measured using an enzyme-linked immunosorbent assay kit (Wuhan Huamei Biological Engineering Co. Ltd., product number: CSB-EL028107HU, detection range: 6.25-400 pg/mL, sensitivity: 1.56 pg/mL).

Statistical analysis

Data were analyzed and processed using SPSS (version 27.0) statistical software. The normality of the data was tested using the Shapiro-Wilk test. Normally distributed data were expressed as mean ± SD and compared using one-way analysis of variance. Non-normally distributed data were expressed as median and interquartile range, and compared using the Wilcoxon rank-sum test. Count data were expressed as percentages and compared using the chi-square test. Spearman correlation analysis was used to analyze the correlation of MAFLD with various indicators as well as the correlation of MAFLD-associated liver fibrosis with ANGPTL8, TyG index, and CRP. Binary logistic regression analysis was used to analyze the risk factors for MAFLD and MAFLD-associated liver fibrosis. Receiver operating characteristic (ROC) curves were used to evaluate the predictive value of ANGPTL8 for the risk of MAFLD and liver fibrosis (FIB-4 ≥ 1.45). Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Clinical characteristics of the study subjects

A flow chart of subject selection is presented in Figure 1, and the clinical characteristics of the study subjects are listed in Table 1. A total of 160 patients were enrolled, including 90 men (56.3%) and 70 women (43.7%). The age of the included population ranged from 18 to 85 years. Among the 160 patients, 80 patients (50%) were diagnosed with MAFLD. Among the patients with MAFLD, 23 patients had liver fibrosis, and 57 patients did not have liver fibrosis, based on the FIB-4 index. The following factors did not significantly differ between the MAFLD and non-MAFLD groups: age, gender, systolic blood pressure, diastolic blood pressure, mean arterial pressure, FPG, and HOMA-β ($P > 0.05$). In contrast, body mass index (BMI), waist-to-hip ratio (WHR), HbA1c, FINS, HOMA-IR, TC, TG, TG/HDL-C ratio, LDL-C/HDL-C ratio, CRP, FIB-4 index, TyG index, and ANGPTL8 level were significantly higher in the MAFLD group than in the non-MAFLD group ($P < 0.05$ for all), while HDL-C was significantly lower in the MAFLD group than in the non-MAFLD group ($P = 0.015$). The LDL-C was also higher in the MAFLD group than in the non-MAFLD group (2.28 ± 0.99 *vs* 2.51 ± 1.05 mmol/L), but the difference was not statistically significant ($P = 0.144$). Finally, the ALT level [14.00 (10.00, 22.75) *vs* 18.00 (12.00, 25.75) U/L] was significantly higher in the MAFLD group ($P = 0.014$), while the AST level did not significantly differ between the 2 groups ($P = 0.181$).

Table 1 Clinical characteristics of all study subjects

	Non-MAFLD group (*n* = 80)	MAFLD group (*n* = 80)	*P* value[a]
Sex (female/male)	37/43	33/47	0.524[1]
Age (yr)	55.35 ± 12.77	54.70 ± 12.15	0.745[2]
WHR	0.89 ± 0.09	0.92 ± 0.07	0.039[2]
BMI (kg/m^2)	22.06 (20.16, 24.64)	24.41 (22.89, 27.61)	< 0.001[3]
SBP (mmHg)	131.00 (121.75, 147.25)	136.00 (125.00, 150.00)	0.193[3]
DBP (mmHg)	83.50 (75.75, 94.00)	87.00 (79.00, 92.00)	0.237[3]
MAP (mmHg)	100.00 (91.00, 112.25)	104.00 (95.00, 111.00)	0.151[3]
ALT (U/L)	14.00 (10.00, 22.75)	18.00 (12.00, 25.75)	0.014[3]
AST (U/L)	18.00 (15.75, 22.00)	19.50 (15.00, 24.00)	0.181[3]
TC (mmol/L)	4.29 (3.55, 4.96)	4.39 (3.61, 5.54)	0.222[3]
TG (mmol/L)	1.04 (0.62, 1.54)	2.69 (1.39, 4.35)	< 0.001[3]
HDL-C (mmol/L)	1.31 (1.04, 1.60)	1.17 (0.85, 1.41)	0.015[3]
LDL-C (mmol/L)	2.28 ± 0.99	2.51 ± 1.05	0.144[2]
TG/HDL-C	0.84 (0.46, 1.33)	2.73 (1.14, 4.24)	< 0.001[3]
LDL-C/HDL-C	1.71 (1.26, 2.29)	2.06 (1.59, 2.92)	0.005[3]
HbA1c (%)	6.00 (5.44, 8.70)	7.00 (6.13, 8.58)	0.018[3]
FPG (mmol/L)	6.58 (5.63, 8.68)	6.67 (5.93, 9.28)	0.370[3]
FINS (μU/mL)	7.01 (4.98, 9.72)	10.00 (5.97, 13.66)	0.004[3]
HOMA-β (%)	50.10 (23.25, 87.63)	55.29 (33.96, 94.46)	0.156[3]
HOMA-IR	2.08 (1.40, 3.34)	2.99 (1.73, 5.50)	0.008[3]
CRP (mg/L)	1.57 (0.98, 2.21)	1.74 (1.19, 2.90)	0.020[3]
ANGPTL8 (pg/mL)	23.95 (16.52, 34.20)	44.93 (33.41, 58.01)	< 0.001[3]
FIB-4 index	1.18 (0.96, 1.46)	1.39 (1.23, 1.76)	< 0.001[3]
TyG index	8.66 ± 0.71	9.56 ± 0.92	< 0.001[2]

[a]*P* < 0.05 is considered significant.
[1]χ^2 test.
[2]*t*-test.
[3]Mann Whitney *U* test.
MAFLD: Metabolic dysfunction-associated fatty liver disease; WHR: Waist-to-hip ratio; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; MAP: Mean arterial pressure; ALT: Alanine transaminase; AST: Aspartate transaminase; TG: Triglyceride; TC: Total cholesterol; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein-cholesterol; HbA1c: Glycated hemoglobin; FPG: Fasting plasma glucose; FINS: Fasting insulin; HOMA-β: Homeostasis model assessment of β-cell function; HOMA-IR: Homeostasis model assessment of insulin resistance; CRP: C-reactive protein; ANGPTL8: Angiopoietin-like protein 8; FIB-4 index: Fibrosis-4 index; TyG index: Triglyceride-glucose index.

Correlation of MAFLD with clinical indicators

Spearman correlation analysis was performed to assess the correlation of the indicators with significant between-group differences with MAFLD. The results are shown in Table 2. Serum ANGPTL8, TyG index, FIB-4 index, BMI, WHR, TG, ALT, FINS, HbA1c, HOMA-IR, CRP, TG/HDL-C, and LDL-C/HDL-C were positively correlated with MAFLD ($P < 0.05$), while HDL-C was negatively correlated with MAFLD ($r = -0.195$, $P = 0.014$). Further, binary logistic regression analysis of the potential risk factors for MAFLD was performed (Table 3). The results showed that ANGPTL8, BMI, and TG had a statistically significant impact on the risk of MAFLD ($P < 0.05$). Serum ANGPTL8 was a risk factor for the occurrence of MAFLD.

Diagnostic performance of ANGPTL8 and TyG index in MAFLD

ROC curves were used to evaluate the diagnostic performance of ANGPTL8, the TyG index, and their combination for the risk of MAFLD (Figure 2). The ROC curve of ANGPTL8 had an area under the curve (AUC) of 0.832 [95% confidence interval (CI): 0.771-0.893, $P < 0.001$], with an optimal cutoff point of 31.18 pg/mL, sensitivity of 80%, and specificity of 73.08%. The ROC curve of the TyG index had an AUC of 0.773 (95%CI: 0.701-0.845, $P < 0.001$), with an optimal cutoff

Table 2 Spearman correlation analysis of metabolic dysfunction-associated fatty liver disease and various indicators

	r	*P* value[a]
ANGPTL8	0.576	< 0.001
TyG index	0.473	< 0.001
FIB-4 index	0.318	< 0.001
BMI	0.394	< 0.001
WHR	0.172	0.031
TG	0.581	< 0.001
HDL-C	-0.195	0.014
ALT	0.195	0.014
FINS	0.231	0.003
HbA1c	0.189	0.017
HOMA-IR	0.211	0.008
CRP	0.189	0.018
TG/HDL-C	0.551	< 0.001
LDL-C/HDL-C	0.226	0.004

[a]$P < 0.05$ is considered significant.

ANGPTL8: Angiopoietin-like protein 8; TyG index: Triglyceride-glucose index; FIB-4 index: Fibrosis-4 index; BMI: Body mass index; WHR: Waist-to-hip ratio; TG: Triglyceride; HDL-C: High-density lipoprotein-cholesterol; ALT: Alanine transaminase; FINS: Fasting insulin; HbA1c: Glycated hemoglobin; HOMA-IR: Homeostasis model assessment of insulin resistance; CRP: C-reactive protein; LDL-C: Low-density lipoprotein-cholesterol.

Table 3 Multifactor logistic regression analysis of factors influencing metabolic dysfunction-associated fatty liver disease

	B	*S.E.*	Wals	*P* value[a]	EXP(B)	95%CI
ANGPTL8	0.116	0.027	19.118	< 0.001	1.123	1.066-1.184
BMI	0.275	0.111	6.142	0.013	1.317	1.059-1.637
ALT	0.032	0.021	2.394	0.122	1.032	0.992-1.075
HDL-C	-0.386	0.804	0.231	0.631	0.680	0.141-3.287
FINS	0.085	0.091	0.875	0.350	1.088	0.911-1.300
HbA1c	-0.248	0.176	1.982	0.159	0.780	0.552-1.102
HOMA-IR	-0.426	0.251	2.869	0.090	0.653	0.399-1.069
CRP	0.160	0.088	3.285	0.070	1.173	0.987-1.395
WHR	-3.793	4.066	0.870	0.351	0.023	0.000-65.098
TG	2.255	0.527	18.277	< 0.001	9.532	3.391-26.800

[a]$P < 0.05$ is considered significant.

CI: Confidence interval; ANGPTL8: Angiopoietin-like protein 8; BMI: Body mass index; ALT: Alanine transaminase; HDL-C: High-density lipoprotein-cholesterol; FINS: Fasting insulin; HbA1c: Glycated hemoglobin; HOMA-IR: Homeostasis model assessment of insulin resistance; CRP: C-reactive protein; WHR: Waist-to-hip ratio; TG: Triglyceride.

point of 9.155, sensitivity of 67.50%, and specificity of 80.77%. The combined ROC curve of ANGPTL8 and the TyG index had an AUC of 0.886 (95%CI: 0.846-0.941, $P < 0.001$), with an optimal cutoff point of 0.546, sensitivity of 75.5%, and specificity of 85.9%.

Clinical characteristics of MAFLD patients with liver fibrosis

According to the FIB-4 index, patients with MAFLD were divided into the non-fibrosis group (FIB-4 < 1.45) and the liver fibrosis group (FIB-4 ≥ 1.45). The serum ANGPTL8 Level (40.75 ± 15.11 *vs* 58.94 ± 12.81 pg/mL, $P < 0.001$) and TyG index (9.38 ± 0.85 *vs* 9.99 ± 0.96, $P = 0.006$) were significantly higher in the liver fibrosis group than in the non-fibrosis group, while serum CRP level [2.13 (1.31, 4.23) *vs* 1.63 (0.98, 1.96) mg/L, $P = 0.034$] was opposite. However, age, gender, systolic

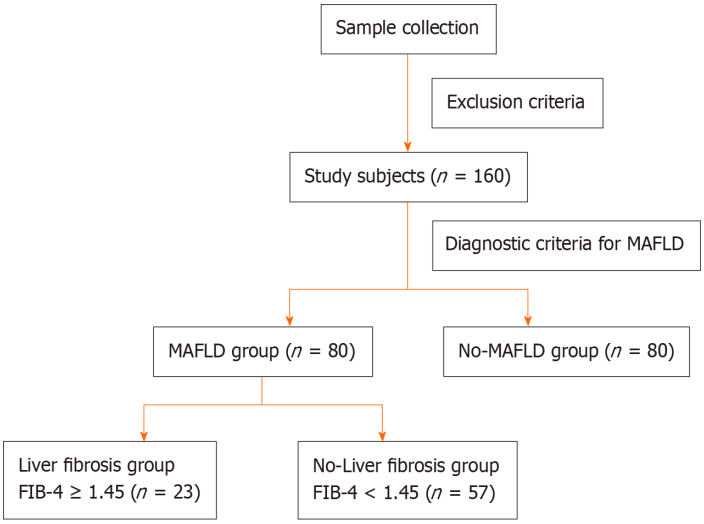

Figure 1 Flow chart of subject selection. MAFLD: Metabolic dysfunction-associated fatty liver disease; FIB-4 index: Fibrosis-4 index.

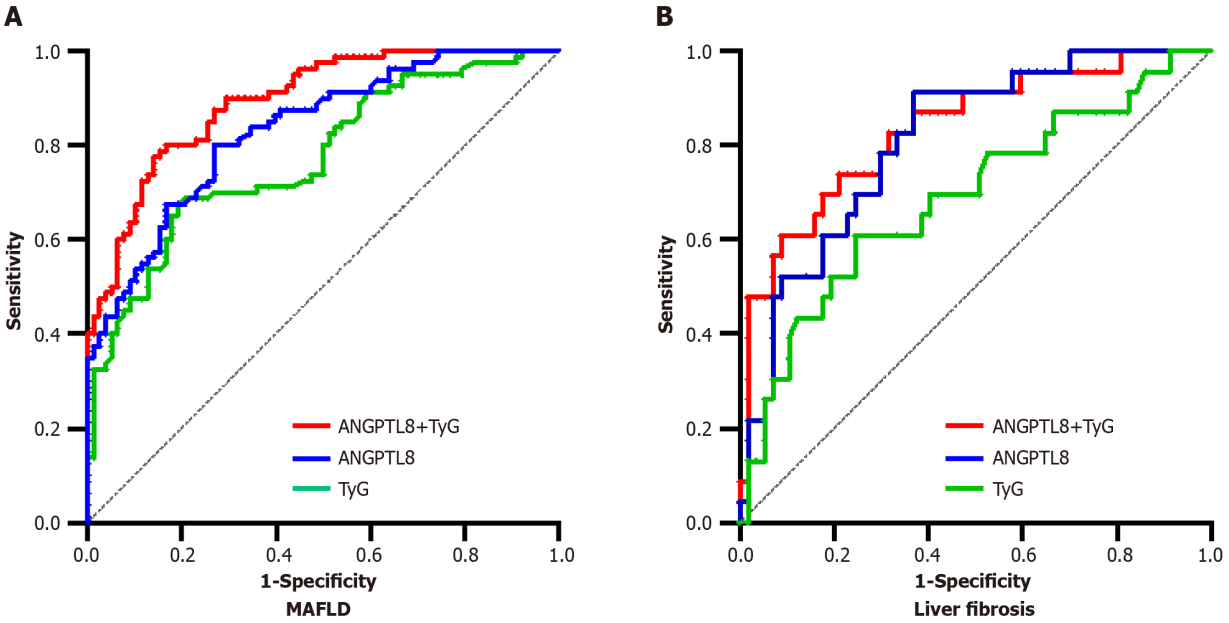

Figure 2 Predictive value of angiopoietin-like protein 8 and triglyceride-glucose index. A: Predictive value in metabolic dysfunction-associated fatty liver disease; B: Predictive value in liver fibrosis. MAFLD: Metabolic dysfunction-associated fatty liver disease; ANGPTL8: Angiopoietin-like protein 8; TyG index: Triglyceride-glucose index.

blood pressure, diastolic blood pressure, mean arterial pressure, FPG, HOMA-β, BMI, WHR, HbA1c, FINS, HOMA-IR, TC, TG, HDL-C, LDL-C, TG/HDL-C, LDL-C/HDL-C, ALT, and AST did no significantly differ between the fibrosis and non-fibrosis groups (all $P > 0.05$, Figure 3).

Correlation of MAFLD-associated liver fibrosis with clinical indicators

Considering the above results, we speculated that there exists a correlation between ANGPTL8 and MAFLD complicated with liver fibrosis. Spearman correlation analysis revealed that liver fibrosis in MAFLD was positively correlated with the serum ANGPTL8 Level ($r = 0.489$, $P < 0.001$) and TyG index ($r = 0.294$, $P = 0.008$), and negatively correlated with the CRP level ($r = -0.238$, $P = 0.033$, Table 4). Furthermore, logistic regression analysis revealed that elevated serum ANGPTL8 level and TyG index were risk factors for liver fibrosis in MAFLD (both $P < 0.05$, Table 5).

Diagnostic performance of ANGPTL8 and TyG index in MAFLD with liver fibrosis

ROC curve analysis was performed to evaluate the predictive value of ANGPTL8, TyG index, and their combination for liver fibrosis in MAFLD (Table 6, Figure 2). The results showed that the AUC of ANGPTL8 was 0.812 (95%CI: 0.713-0.910, $P < 0.001$), with an optimal cutoff point of 44.36 pg/mL, sensitivity of 91.3%, and specificity of 61.36%. The AUC of TyG index was 0.688 (95%CI: 0.553-0.822, $P < 0.001$), with an optimal cutoff point of 9.925, sensitivity of 60.87%, and specificity

Table 4 Spearman correlation of metabolic dysfunction-associated fatty liver disease complicated with liver fibrosis with serum angiopoietin-like protein 8, C-reactive protein, and triglyceride-glucose index

	r	*P* value[a]
ANGPTL8	0.489	< 0.001
TyG index	0.294	0.008
CRP	-0.238	0.033

[a]*P* < 0.05 is considered significant.
ANGPTL8: Angiopoietin-like protein 8; CRP: C-reactive protein; TyG index: Triglyceride-glucose index.

Table 5 Logistical analysis of metabolic dysfunction-associated fatty liver disease with liver fibrosis

	B	*S.E.*	Wals	*P* value[a]	EXP(B)	95%CI
ANGPTL8	0.089	0.023	14.513	< 0.001	1.093	1.044-1.144
TyG index	0868	0.350	6.146	0.013	2.383	1.199-4.736
CRP	-0.560	0.302	3.449	0.063	0.571	0.316-1.032

[a]*P* < 0.05 is considered significant.
CI: Confidence interval; ANGPTL8: Angiopoietin-like protein 8; TyG index: Triglyceride-glucose index; CRP: C-reactive protein.

Table 6 Receiver operating characteristic curve analysis of the predictive value of angiopoietin-like protein 8, triglyceride-glucose index, and its combination with triglyceride-glucose index for metabolic dysfunction-associated fatty liver disease and liver fibrosis

	AUC	95%CI	Cutoff	Sensitivity	Specificity	*P* value[a]
Prediction of MAFLD						
ANGPTL8	0.832	0.771-0.893	31.18	80.00	73.08	< 0.001
TyG index	0.773	0.701-0.845	9.155	67.5	80.77	< 0.001
ANGPTL8 + TyG index	0.886	0.846-0.941	0.546	75.5	85.9	< 0.001
Prediction of MAFLD with liver fibrosis						
ANGPTL8	0.812	0.713-0.910	44.36	91.30	61.36	< 0.001
TyG index	0.688	0.553-0.822	9.925	60.87	75.44	< 0.001
ANGPTL8 + TyG index	0.835	0.735-0.935	0.3897	73.91	78.95	< 0.001

[a]*P* < 0.05 is considered significant.
AUC: Area under the curve; MAFLD: Metabolic dysfunction-associated fatty liver disease; CI: Confidence interval; ANGPTL8: Angiopoietin-like protein 8; TyG index: Triglyceride-glucose index.

of 75.44%. The AUC of their combination was 0.835 (95%CI: 0.735-0.935, $P < 0.001$), with an optimal cutoff point of 0.3897, sensitivity of 73.91%, and specificity of 78.95%.

DISCUSSION

The data from this study indicate that serum ANGPTL8 is significantly elevated in MAFLD patients and is closely associated with the risk of MAFLD and MAFLD-associated liver fibrosis. As serum ANGPTL8 levels increase, the risks of MAFLD and liver fibrosis also increase. Hence, serum ANGPTL8 may be a powerful indicator for predicting the risk of MAFLD and MAFLD-associated liver fibrosis. The combination of serum ANGPTL8 and the TyG index may have an even higher predictive value for the risk of MAFLD and the presence of liver fibrosis.

MAFLD is a progressive liver disease that can lead to steatohepatitis, liver fibrosis, cirrhosis, and even hepatocellular carcinoma. Its pathophysiology is complex and involves many interconnected processes, including metabolic dysregulation, lipotoxicity, insulin resistance, chronic inflammation, oxidative stress, mitochondrial autophagy, and gut microbiota[11-13]. Currently, MAFLD has become an increasingly common factor in end-stage liver disease and

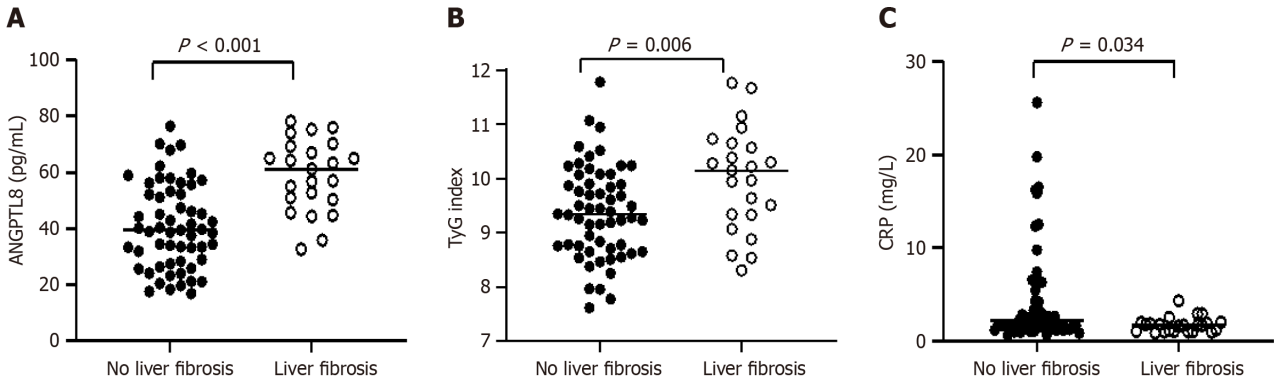

Figure 3 Comparison of 3 indexs between the liver fibrosis and non-fibrosis groups. A: Angiopoietin-like protein 8; B: Triglyceride-glucose index levels; C: C-reactive protein. MAFLD: Metabolic dysfunction-associated fatty liver disease; ANGPTL8: Angiopoietin-like protein 8; CRP: C-reactive protein.

cardiovascular disease, posing a significant economic burden on public health[14,15].

Studies have suggested that ANGPTL8, as a novel protein secreted by the liver or adipose tissue, plays a dual role in lipid and glucose metabolism. The latest research has proposed a dual mode of ANGPTL8 regulation of lipoprotein lipase (LPL), known as the "ANGPTL3-4-8" model[16]. ANGPTL8 can inhibit LPL activity; control postprandial TG flux, and lipid uptake and storage in brown adipose tissue through synergistic interactions with ANGPTL3 and ANGPTL4; reduce TG clearance; and ultimately lead to a significant increase in serum TG levels[17]. Animal experiments also support a positive correlation between ANGPTL8 and TG levels; knockout of ANGPTL8 leads to decreased lipid content in adipocytes[18], which can significantly improve lipid uptake and reduce lipid deposition[4]. It is worth noting that ANGPTL8 is also associated with TC, HDL-C, and LDL-C[19]. Serum ANGPTL8 can also improve insulin resistance by directly activating insulin-mediated AKT phosphorylation[20] or regulating the PI3K/Akt signaling pathway to enhance insulin sensitivity[21], promote glycogen synthesis, and inhibit gluconeogenesis. All of the above findings indicate that ANGPTL8 is an important link in glucose and lipid metabolism, and may be involved in the development of MAFLD.

Research has shown that the ANGPTL8 level is significantly increased in patients with MAFLD[22], and it is positively correlated with the lipid content in liver cells[23]. In a mouse experiment, it was discovered that inhibiting serum ANGPTL8 levels with antisense oligonucleotides can suppress hepatic steatosis in high-fat-fed mice[4]. This further suggests that ANGPTL8 may play an important role in the occurrence and development of MAFLD. ANGPTL8 expression is increased in the livers of MAFLD patients, which is possibly related to endoplasmic reticulum stress in liver cells[24]. ANGPTL8 can also induce the expression of inflammatory factors by regulating the NF-kB signaling pathway [25], thereby participating in the inflammatory response and promoting the progression of MAFLD to liver fibrosis and cirrhosis. The present study also found similar results by comparing the clinical characteristics of the non-MAFLD and MAFLD populations. Serum ANGPTL8 was positively correlated with the risk of MAFLD, and as serum ANGPTL8 levels increased, the risk of MAFLD also increased. Logistic regression analysis showed that ANGPTL8 is a risk factor for MAFLD. The TyG index, which reflects insulin resistance, has been proven to be of significant value in predicting the risk of MAFLD and cardiovascular risk factors such as hypertension and atherosclerosis. In our study, we also found a close correlation between the TyG index and the risk of MAFLD. We further constructed ROC curves for predicting MAFLD by using ANGPTL8 alone and in combination with the TyG index, and obtained AUCs of 0.832 and 0.886, respectively. This indicates that ANGPTL8 can serve as a predictive factor for the risk of MAFLD, and its combination with the TyG index may have an even higher predictive value for the risk of MAFLD.

FIB-4 is a widely used non-invasive index for assessing liver fibrosis, with diagnostic efficacy comparable to the gold standard liver biopsy[26]. Studies have proposed that a FIB-4 value of < 1.45 can exclude progressive fibrosis, and this has been widely validated[9,10]. ANGPTL8 can promote the occurrence and development of MAFLD by participating in glucose and lipid metabolism, insulin resistance, and energy homeostasis[22,27], but the relationship between ANGPTL8 and liver fibrosis remains controversial. In 2016, Cengiz *et al*[28] found that serum ANGPTL8 levels were lower in NAFLD patients than in healthy individuals, and higher in NAFLD patients with mild fibrosis than in patients with significant fibrosis. However, some authors have reported contradictory conclusions. Ke *et al*[22] reported that the circulating level of ANGPTL8 is significantly higher in MAFLD patients than in healthy individuals, and is not affected by race, region, BMI, *etc*.; they concluded that elevated serum ANGPTL8 is positively correlated with MAFLD. In addition, Zhang *et al*[29] suggested that ANGPTL8 can act as a pro-inflammatory factor, and promote the development of liver fibrosis. In our study, we found similar results: ANGPTL8 levels were significantly increased in MAFLD patients with liver fibrosis (FIB-4 ≥ 1.45), and were positively correlated with MAFLD combined with liver fibrosis. Hence, we concluded that elevated serum ANGPTL8 levels are associated with an increased risk of liver fibrosis in MAFLD patients.

Studies have also shown a significant correlation between the TyG index and the severity of hepatic steatosis and liver fibrosis in NAFLD patients[30]. Elevated TyG index is an independent predictor of MAFLD and is positively correlated with the MAFLD fibrosis score and FIB-4 index, suggesting that a significant increase in the TyG index may indicate the development of liver fibrosis[31]. In 113 patients with histologically confirmed NAFLD, the TyG index levels were significantly higher in the F3 fibrosis stage than in the F0–F1 stage ($P < 0.0001$)[32]. However, Guo *et al*[30] found that the AUC value of the TyG index for predicting NAFLD was 0.761, while its AUC value for predicting liver fibrosis was 0.589, indicating that the TyG index has a relatively low accuracy for predicting NAFLD-associated liver fibrosis. In our study,

we constructed ROC curves to analyze whether serum ANGPTL8 as well as the combination of serum ANGPTL8 and the TyG index could predict the coexistence of liver fibrosis with MAFLD, and obtained AUC values of 0.812 and 0.835, respectively. Hence, serum ANGPTL8 is a powerful predictor of the coexistence of liver fibrosis with MAFLD, and the combined use of serum ANGPTL8 and the TyG index may improve the predictive efficacy for liver fibrosis in MAFLD.

This study has the following limitations: First, the gold standard for the diagnosis of MAFLD and liver fibrosis is liver biopsy, but this study only used imaging examinations and the FIB-4 index due to limitations in conditions. Second, this study had a small sample size, and the results may be biased and require further validation with a larger sample size. Third, this study is a cross-sectional study and cannot reflect the specific mechanisms of ANGPTL8 in MAFLD. Further prospective studies are needed to explore and clarify these mechanisms.

CONCLUSION

In conclusion, the study reveals that serum levels of ANGPTL8 are elevated in patients diagnosed with MAFLD, and notably higher in those with concurrent liver fibrosis. The close correlation of serum ANGPTL8 with the risks associated with MAFLD and the progression of liver fibrosis underscores its significance. Serving as a predictive marker for both MAFLD risks and its associated liver fibrosis, serum ANGPTL8 exhibits enhanced predictive value when combined with the TyG index. Consequently, we posit that serum ANGPTL8 stands as a valuable and straightforward reference marker for the early prediction and timely management of MAFLD, enriching the repertoire of non-invasive assessment methods in clinical settings. Monitoring changes in ANGPTL8 levels facilitates early detection of MAFLD-associated liver fibrosis, enabling prompt intervention and management strategies to mitigate disease progression and reduce the risk of mortality.

ARTICLE HIGHLIGHTS

Research background

Metabolic dysfunction-associated fatty liver disease (MAFLD) is now recognized as a prevalent global chronic liver disorder, standing as the primary contributor to end-stage liver disease and cardiovascular complications. This condition imposes a significant economic burden on public health. Previous research has elucidated the pivotal role of angiopoietin-like protein 8 (ANGPTL8) in the pathogenesis and progression of MAFLD, showcasing variations across different disease stages. Understanding ANGPTL8's involvement in MAFLD development informs early intervention strategies and aids in reducing mortality.

Research motivation

This study was motivated by the need for a convenient laboratory indicator facilitating the early identification of MAFLD and dynamic monitoring of its progression. Providing early intervention strategies for MAFLD patients is crucial to delay disease progression and reduce mortality.

Research objectives

The primary objective of this study was to conduct a comparative analysis of serum ANGPTL8 levels between individuals with and without MAFLD, investigating the predictive value of ANGPTL8 in relation to MAFLD development and progression.

Research methods

In this cross-sectional study, 160 patients were enrolled, with 80 (50%) diagnosed with MAFLD. MAFLD patients were further categorized into hepatic fibrosis and non-hepatic fibrosis groups based on the fibrosis-4 index. Logistic regression analysis and receiver operating characteristic curves were employed to explore the impact and predictive ability of serum ANGPTL8.

Research results

Compared with the non-MAFLD group, serum ANGPTL8 levels and the triglyceride-glucose (TyG) index were significantly elevated in the MAFLD group, positively correlated with MAFLD. The combined ANGPTL8 and TyG index showed high predictive accuracy for MAFLD. Similarly, in the liver fibrosis group, both ANGPTL8 and the TyG index were significantly increased, positively correlated with liver fibrosis, with robust predictive accuracy for MAFLD-associated liver fibrosis.

Research conclusions

Serum ANGPTL8 appears pivotal in the pathogenesis and progression of MAFLD, emerging as a potential biomarker for predicting both MAFLD and its associated hepatic fibrosis. The combined assessment of serum ANGPTL8 levels and the TyG index enhances predictive accuracy. Understanding the underlying mechanisms linking ANGPTL8 with MAFLD provides valuable insights for early diagnosis, risk stratification, and timely intervention, ultimately alleviating the burden of this disease.

Research perspectives

Future studies should consider expanding sample sizes and incorporating liver biopsy and other methods to distinguish MAFLD-related liver fibrosis. Longitudinal studies are necessary to analyze serum ANGPTL8 changes at each MAFLD stage, confirming the relationship between serum ANGPTL8 and the occurrence and progression of MAFLD.

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FOOTNOTES

Author contributions: Gan LL participated in the study design and wrote the manuscript; Yan YM conducted the design of the study and reviewed/edited the drafts, and is guarantor; Gan LL, Zhu X, Xia C, Gao Y and Wu WC collected and analyzed the data; Li Q, Li L and Dai Z revised the manuscript. All authors contributed to the article and approved the submitted article.

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Retrospective Study

Myosteatosis is associated with coronary artery calcification in patients with type 2 diabetes

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Abstract

BACKGROUND

Myosteatosis, rather than low muscle mass, is the primary etiologic factor of sarcopenia in patients with type 2 diabetes mellitus (T2DM). Myosteatosis may lead to a series of metabolic dysfunctions, such as insulin resistance, systematic inflammation, and oxidative stress, and all these dysfunctions are closely associated with the acceleration of T2DM and atherosclerosis.

AIM

To investigate the association between myosteatosis and coronary artery calcification (CAC) in patients with T2DM.

METHODS

Patients with T2DM, who had not experienced major cardiovascular events and had undergone both abdominal and thoracic computed tomography (CT) scans, were included. The mean skeletal muscle attenuation was assessed using abdominal CT images at the L3 level. The CAC score was determined from thoracic CT images using the Agatston scoring method. Myosteatosis was diagnosed according to Martin's criteria. Severe CAC (SCAC) was defined when

the CAC score exceeded 300. Logistic regression and decision tree analyses were performed.

RESULTS

A total of 652 patients with T2DM were enrolled. Among them, 167 (25.6%) patients had SCAC. Logistic regression analysis demonstrated that myosteatosis, age, duration of diabetes, cigarette smoking, and alcohol consumption were independent risk factors of SCAC. Myosteatosis was significantly associated with an increased risk of SCAC (OR = 2.381, P = 0.003). The association between myosteatosis and SCAC was significant in the younger patients (OR = 2.672, 95%CI: 1.477-4.834, P = 0.002), but not the older patients (OR = 1.456, 95%CI: 0.863-2.455, P = 0.188), and was more prominent in the population with lower risks of atherosclerosis. The decision tree analyses prioritized older age as the primary variable for SCAC. In older patients, cigarette smoking was the main contributing factor for SCAC, while in younger patients, it was myosteatosis.

CONCLUSION

Myosteatosis is a novel risk factor for atherosclerosis in patients with T2DM, especially in the population with younger ages and fewer traditional risk factors.

Key Words: Type 2 diabetes; Myosteatosis; Muscle quality; Coronary artery calcification; Atherosclerosis; Cardiovascular diseases

Core Tip: Myosteatosis, rather than low muscle mass, is the primary etiologic factor of sarcopenia in patients with type 2 diabetes mellitus (T2DM). Myosteatosis may lead to a series of metabolic dysfunctions that are closely associated with the acceleration of T2DM and atherosclerosis. This study demonstrated that myosteatosis was a novel risk factor for atherosclerosis in patients with T2DM, especially in the population with younger ages and fewer traditional risk factors. Therefore, this indicates the potential benefit of initiating muscle-strengthening exercises and improving muscle quality at a younger age.

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INTRODUCTION

The prevalence of diabetes, especially type 2 diabetes mellitus (T2DM), has been dramatically increasing in China, from 10.9% in 2013 to 12.4% in 2018, and atherosclerotic cardiovascular disease is the leading cause of mortality in these patients[1,2]. Myosteatosis, a marker of muscle quality, has been proposed as a novel risk factor for atherosclerotic cardiovascular diseases, independent of muscle mass[3-6]. Myosteatosis may lead to a series of metabolic dysfunctions, such as insulin resistance, systematic inflammation, and oxidative stress, and all these dysfunctions are closely associated with the acceleration of T2DM and atherosclerosis (Supplementary Figure 1)[3,7,8].

Computed tomography (CT) is considered the gold standard for myosteatosis measurement, and lower muscle radiodensity indicates higher fat infiltration (*i.e.*, myosteatosis)[9]. Recently, a large-sample study involving 20986 participants indicated that the patients with T2DM had significantly higher values of muscle mass but significantly lower values of muscle quality[10,11]. Therefore, low muscle quality rather than low muscle mass is the major characteristic change of skeletal muscle in patients with T2DM. Patients with T2DM have high risks of myosteatosis and atherosclerosis. However, the association between myosteatosis and coronary artery calcification (CAC) in this population has not been reported yet.

CAC score (CACS), which can be calculated with the Agatston scoring method, is considered a useful tool for identifying coronary atherosclerosis. The risk of coronary events in patients with CACS > 300 across various ethnic groups has a nearly 10-fold increase[12-14]. In Australia, CACS is used to help define the risk in the primary prevention of cardiovascular diseases[15]. The long-term (> 10 years) prognostic value of CACS in cardiovascular diseases has also been validated in patients with T2DM[16].

Herein, we performed this cross-sectional study to analyze the association of myosteatosis with CAC in patients with T2DM. The myosteatosis and CACS were evaluated with abdominal and thoracic CT, respectively.

MATERIALS AND METHODS

Study population

Patients with T2DM who were hospitalized in the Department of Endocrinology, Affiliated Hospital of Jining Medical University between January 2017 and December 2021 were included in this study. They all underwent abdominal and thoracic CT scans. The exclusion criteria included: (1) Patients with age < 30 or > 80 years old; (2) patients with a history of major cardiovascular events (*i.e.*, myocardial infarction, congestive heart failure, coronary stent implantation, and cerebrovascular accidents); and (3) patients with consumptive or critical diseases (*i.e.*, malignant tumors, abnormal thyroid function, and stage V diabetic nephropathy). At admission, all patients were informed that their medical records may be used for research purposes unless they indicate their opposition. For the present study, no patient indicated opposition. This study was approved by the Ethics Committee of the Affiliated Hospital of Jining Medical University (No. 2021-08-C001).

Laboratory measurements and assessment of diabetic complications

All biochemical and immune indexes were measured in the laboratory of our hospital. Fasting glucose and C-peptide were measured for calculating homeostasis model assessment 2 of insulin resistance and homeostasis model assessment 2 of beta-cell function (HOMA2-β).

Measurement of body composition and CAC

Both abdominal and thoracic CT scans were performed using a Dual-Source Flash CT scanner (Siemens, Erlangen, Germany). The body composition was assessed using abdominal axial CT images at the L3 level and the Slice-O-Matic software (V.5.0, TomoVision, Montreal, Quebec, Canada), as described in our previous study[17]. The CT attenuation thresholds were from -29 to 150 Hounsfield Unit (HU) for skeletal muscle, from -150 to -30 HU for visceral adipose tissue, and from -190 to -30 HU for intramuscular and subcutaneous adipose tissue[18]. The mean skeletal muscle attenuation (MMA), which was automatically calculated by the software, was shown as the mean radiation attenuation of skeletal muscle in HU. Myosteatosis was diagnosed according to Martin's criteria, *i.e.* MMA < 33 HU with body mass index (BMI) ≥ 25 kg/m^2 or MMA < 41 HU with BMI < 25 kg/m^2[19]. The skeletal muscle index (SMI) (cm^2/m^2) was calculated by normalizing the L3 cross-sectional skeletal muscle area in cm^2 to height in m^2[20]. The fat mass index (kg/m^2), which is proposed by VanItallie *et al*[21] and is an indicator of nutritional status, was calculated by normalizing fat mass in kg to height in m^2[21,22]. The fat mass was calculated with the following formula: fat mass (kg) = 0.042 × (total adipose area at L3 in cm^2) + 11.2[22]. The CACS was calculated based on the thoracic CT images by the automated software of syngo *via* and with the Agatston method. Severe CAC (SCAC) was defined when the CACS was > 300[14].

Definitions and diagnosis

Coronary heart disease (CHD) was defined as a suspected history of CHD confirmed through CT coronary angiography. Hypertension was defined as systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, and/or use of antihypertensive medications. Dyslipidemia was defined as disorders of lipoprotein metabolism and/or the use of lipid medications. Alcohol consumption was defined as consuming at least 30 g of alcohol per week for at least a year. Cigarette smoking was defined as smoking at least 100 cigarettes in a lifetime[23]. Diabetic complications were assessed systematically according to the guidelines for the prevention and control of T2DM in China[24]. Diabetic nephropathy was diagnosed when there was elevated urinary albumin excretion and reduced estimated glomerular filtration rate in the absence of other primary causes of kidney damage. Diabetic peripheral neuropathy referred to the symptoms or signs of peripheral nerve dysfunction in diabetic patients that cannot be attributed to other causes. Asymptomatic patients must be diagnosed by physical examination or neuro-electrophysiological examination. Diabetic retinopathy was diagnosed by an ophthalmologist who specialized in diabetic retinopathy, according to the international clinical grading standard for diabetic retinopathy. Lower-extremity arterial disease was diagnosed if the patients had a resting ankle-brachial index (ABI) ≤ 0.90. For patients who experienced discomfort upon moving and had a resting ABI ≥ 0.90, lower-extremity arterial disease was also diagnosed if the ABI decreased by 15%-20% after a treadmill test.

Statistical analysis

Continuous variables with normal distribution are presented as mean ± standard deviation, whereas those with non-normal distribution are presented as median and interquartile range. Categorical variables are described by the number and percentage. The characteristics of the study population were compared using independent samples *t*-test, Mann-Whitney *U* test, or χ^2 test, as appropriate. The variables with statistical significance between the two groups were enrolled in the logistic regression analysis to identify independent factors for SCAC. Receiver operating characteristic (ROC) curves were plotted and the area under the curve (AUC) of independent factors for SCAC was compared using the *z*-test. The Youden index was calculated to determine the cut-off points of age in distinguishing SCAC. Subgroups were stratified based on the risk factors of atherosclerosis. The Chi-squared Automatic Interaction Detection (CHAID) decision tree analysis was further performed based on the identified independent factors. The minimum parent and child nodes were determined as 100 and 50, respectively. Statistical analysis was performed using SPSS software (V.26.0). The two-sided *P* value less than 0.05 was considered statistically significant.

RESULTS

Characteristics of the study population

A total of 652 patients with T2DM were enrolled in this study. The characteristics of the study population are presented in Table 1. There were 425 (65.2%) males and 227 (34.8%) females. Among the 652 patients, 167 (25.6%) had SCAC and were classified into the T2DM + SCAC group. The remaining patients were classified into the T2DM group. Patients in the T2DM + SCAC group had higher values of age, diabetes duration, fasting glucose, creatinine, blood urea nitrogen, and cystatin C; had higher percentages of myosteatosis, CHD, cigarette smoking, alcohol consumption, aspirin usage, hypertension, diabetic nephropathy, and diabetic retinopathy; and received more types of antidiabetics, lipid-lowering, and antihypertensive drugs. However, they had lower values of hemoglobin, alanine transaminase, low-density lipoprotein, free triiodothyronine, and SMI. The comparison of clinical characteristics of patients with and without myosteatosis is presented in the Supplementary Table 1.

Role of myosteatosis in predicting SCAC

The patients with myosteatosis exhibited significantly higher percentages of SCAC compared with those without myosteatosis (35.6% *vs* 16.6%). Logistic regression analysis revealed that myosteatosis, age, duration of diabetes, cigarette smoking, and alcohol consumption were independent risk factors for SCAC (Figure 1). Patients with myosteatosis showed an increased risk of SCAC (OR = 2.381, 95%CI: 1.347-4.207, $P = 0.003$) after adjustment for age, diabetes duration, cigarette smoking, and alcohol consumption.

The predictive abilities of the aforementioned five factors for SCAC were evaluated using ROC curve analysis (Figure 2). Age had the highest AUC, followed by duration of diabetes, myosteatosis, cigarette smoking, and drinking. The combined model of the five independent risk factors yielded a higher AUC than age alone (0.794 *vs* 0.734, $P = 0.034$).

Subgroup analysis

Given the variation in age-specific risk of cardiovascular disease by gender, ROC curve analyses were conducted to determine the cut-off points of age in predicting SCAC. The cut-off points for older age were identified as age > 56.5 years in males and age > 63.5 years in females (Figure 3). Patients in the older age group exhibited significantly higher percentages of SCAC compared to those in the younger age group (47.3% *vs* 13.2% in males and 38.7% *vs* 13.4% in females).

Subgroup stratification based on sex, age, BMI, cigarette smoking, alcohol consumption, dyslipidemia, and hypertension was performed (Figure 4). The association between myosteatosis and SCAC was found to be significant in younger patients (OR = 2.672, 95%CI: 1.477-4.834, $P = 0.002$) rather than in older patients (OR = 1.456, 95%CI: 0.863-2.455, $P = 0.188$), and was more prominent in patients with a lower risk of atherosclerosis, such as BMI < 25 kg/m^2, without cigarette smoking, alcohol consumption, dyslipidemia, and hypertension.

Construction of CHAID decision tree

CHAID decision tree analysis was conducted using the older age, myosteatosis, and other significantly different factors between the T2DM + SCAC and T2DM groups. Older age, myosteatosis, and cigarette smoking were determined as critical variables and were included in the construction of the CHAID decision tree (Figure 5). The primary variable for SCAC was older age (OR = 5.186, 95%CI: 3.543-7.590, $P < 0.001$). Among patients of older age, the primary factor was cigarette smoking (OR = 2.459, 95%CI: 1.486-4.069, $P < 0.001$), while among younger patients, the primary factor was myosteatosis (OR = 2.672, 95%CI: 1.477-4.834, $P = 0.001$).

DISCUSSION

To the best of our knowledge, this is the first study to investigate the relationship of myosteatosis with CAC in patients with T2DM. Logistic regression and CHAID decision tree analyses confirmed that myosteatosis, age, cigarette smoking, and alcohol consumption were independent factors of SCAC. Moreover, the association between myosteatosis and CAC might be more prominent in the younger population.

Two large-sample cross-sectional studies have investigated the relationship of muscle quality with CAC in populations other than T2DM[25]. The Coronary Artery Risk Development in Young Adults study enrolled 3051 participants aged 43 to 55 years and defined CAC with CACS > 0[25]. Compared with those with the lowest quartile, the young adults with the upper quartile of abdominal intermuscular adipose tissue volume had a higher risk of CAC [OR 1.6 (1.2-2.1)] after adjusting for cardiovascular disease risk factors[25]. In another study by Lee *et al*[4], a total of 4068 subjects without cardiovascular diseases were included and significant CAC was defined if CACS was > 100. They found that the higher ratio of the muscle area with normal attenuation to the total abdominal muscle area was strongly associated with a lower prevalence of significant CAC after adjustment[4]. Different from these two studies, our study focused on patients with T2DM, and this population is associated with high risks of both myosteatosis and CAC. We demonstrated that myosteatosis was significantly associated with SCAC in patients with T2DM, independent of traditional cardiovascular disease risk factors.

In our study, we found that in addition to myosteatosis, factors such as age, duration of diabetes, smoking, and drinking[26-28] were identified as independent risk factors for SCAC. It is worth noting that the age-specific risk of cardiovascular disease varies by gender, being significantly lower in women before menopause[29,30]. We determined

Table 1 Characteristics of the study population

Variables	All patients (*n* = 652)	T2DM group (*n* = 485)	T2DM + SCAC group (*n* = 167)	*P* value
Male (%)	425 (65.2)	312 (64.3)	113 (67.7)	0.453
Age (yr)	55.95 ± 10.87	53.75 ± 10.62	62.34 ± 8.94	< 0.001
Diabetes duration (yr)	8.97 ± 7.13	7.87 ± 6.36	12.15 ± 8.22	< 0.001
Body mass index (kg/m^2)	25.79 ± 3.66	25.87 ± 3.61	25.56 ± 3.79	0.358
Fasting glucose (mmol/L)	7.67 ± 2.23	7.53 ± 2.19	8.09 ± 2.31	0.018
Fasting C peptide (ng/mL)	2.27 ± 1.06	2.25 ± 0.97	2.32 ± 1.30	0.581
Hemoglobin A1c (%)	8.71 ± 2.19	8.76 ± 2.23	8.59 ± 2.06	0.384
HOMA2-β	166.51 ± 93.75	169.89 ± 92.31	155.97 ± 97.78	0.170
HOMA2-IR	5.62 ± 2.67	5.55 ± 2.48	5.84 ± 3.20	0.314
Hemoglobin (g/L)	138.69 ± 21.98	139.77 ± 22.44	135.52 ± 20.30	0.032
Albumin (g/L)	43.14 ± 4.51	43.34 ± 4.31	42.55 ± 5.01	0.071
Alanine transaminase (U/L)	18.20 (13.30, 27.80)	19.35 (13.93, 28.90)	15.90 (12.20, 21.90)	< 0.001
Creatinine (mg/L)	61.38 ± 16.65	60.30 ± 15.78	64.49 ± 18.66	0.010
Blood urea nitrogen (mg/dL)	5.65 ± 1.59	5.52 ± 1.45	6.04 ± 1.90	< 0.001
Cystatin C (mg/L)	1.00 ± 0.29	0.97 ± 0.25	1.11 ± 0.35	< 0.001
Triglycerides (mmol/L)	1.44 (0.98, 2.24)	1.44 (0.98, 2.32)	1.39 (0.95, 1.99)	0.199
Total cholesterol (mmol/L)	4.63 ± 1.56	4.70 ± 1.33	4.44 ± 2.09	0.059
HDL (mmol/L)	1.18 ± 0.38	1.18 ± 0.40	1.16 ± 0.33	0.506
LDL (mmol/L)	2.77 ± 1.18	2.86 ± 1.22	2.53 ± 1.05	0.002
FT3 (pmol/L)	4.53 ± 1.40	4.61 ± 1.57	4.30 ± 0.71	0.016
FT4 (pmol/L)	16.58 ± 3.04	16.57 ± 3.08	16.59 ± 2.91	0.943
TSH (pmol/L)	2.25 ± 1.41	2.24 ± 1.37	2.30 ± 1.54	0.597
SBP (mmHg)	136.23 ± 19.06	136.10 ± 19.82	136.63 ± 16.72	0.736
DBP (mmHg)	81.13 ± 13.00	81.19 ± 13.43	80.95 ± 11.68	0.840
MMA (HU)	36.41 ± 7.29	37.24 ± 7.23	34.02 ± 6.95	< 0.001
Myosteatosis (%)	309 (47.4)	199 (41.0)	110 (65.9)	< 0.001
CHD (%)	166 (25.5)	96 (19.8)	70 (41.9)	< 0.001
SMI (cm^2/m^2)	46.71 ± 9.31	47.18 ± 9.30	45.34 ± 9.23	0.027
FMI (kg/m^2)	8.56 ± 1.79	8.51 ± 1.79	8.68 ± 1.79	0.290
Cigarette smoking (%)	261 (40.0)	170 (35.1)	91 (54.5)	< 0.001
Alcohol intake (%)	295 (45.2)	204 (42.1)	91 (54.5)	0.007
Dyslipidemia (%)	369 (56.6)	282 (58.1)	87 (52.1)	0.176
Hypertension (%)	308 (47.2)	215 (44.3)	93 (55.7)	0.012
Diabetic complications (%)	564 (86.5)	410 (84.5)	154 (92.2)	0.012
DN (%)	247 (37.9)	169 (34.8)	78 (46.7)	0.007
DPN (%)	498 (76.4)	362 (74.6)	136 (81.4)	0.091
LEAD (%)	105 (16.1)	73 (15.1)	32 (19.2)	0.223
DR (%)	185 (28.4)	118 (24.3)	67 (40.1)	< 0.001
Antidiabetics (%)	569 (87.3)	411 (84.7)	158 (94.6)	0.001
Insulin (%)	249 (38.2)	172 (35.5)	77 (46.1)	0.016

Metformin (%)	434 (66.6)	310 (63.9)	124 (74.3)	0.017
Sulphonylureas (%)	312 (47.9)	230 (47.7)	82 (49.1)	0.720
Acarbose (%)	257 (39.4)	180 (37.1)	77 (46.1)	0.044
Others (%)	167 (25.6)	120 (24.7)	47 (28.1)	0.411
Lipid-lowering drugs (%)	140 (21.5)	84 (17.3)	56 (33.5)	< 0.001
Statins (%)	131 (20.1)	75 (15.5)	56 (33.5)	< 0.001
Fibrates (%)	9 (1.4)	9 (1.9)	0 (0.0)	0.121
Antihypertensive drugs (%)	237 (36.3)	158 (32.6)	79 (47.3)	0.001
ACE inhibitors (%)	33 (5.1)	20 (4.1)	13 (7.8)	0.068
ARBs (%)	105 (16.1)	71 (14.6)	34 (20.4)	0.088
Calcium antagonists (%)	134 (20.6)	89 (18.4)	45 (26.9)	0.020
β-Blockers (%)	64 (9.8)	36 (7.4)	28 (16.8)	0.001
Diuretics (%)	32 (4.9)	22 (4.5)	10 (6.0)	0.533
Aspirin (%)	145 (22.2)	84 (17.3)	61 (36.5)	< 0.001

T2DM: Type 2 diabetes mellitus; SCAC: Severe coronary artery calcification; HOMA2-β: Homeostasis model assessment 2 of beta-cell function; HOMA2-IR: Homeostasis model assessment 2 of insulin resistance; HDL: High-density lipoproteins; LDL: Low-density lipoproteins; FT3: Free triiodothyronine; FT4: Free thyroxine; TSH: Thyroid-stimulating hormone; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; CHD: Coronary heart disease; SMI: Skeletal muscle index; MMA: Mean skeletal muscle attenuation; FMI: Fat mass index; DN: Diabetic nephropathy; DPN: Diabetic peripheral neuropathy; LEAD: Lower extremity arterial disease; DR: Diabetic retinopathy; ACE: Angiotensin-converting enzyme; ARB: Angiotensin II receptor blocker.

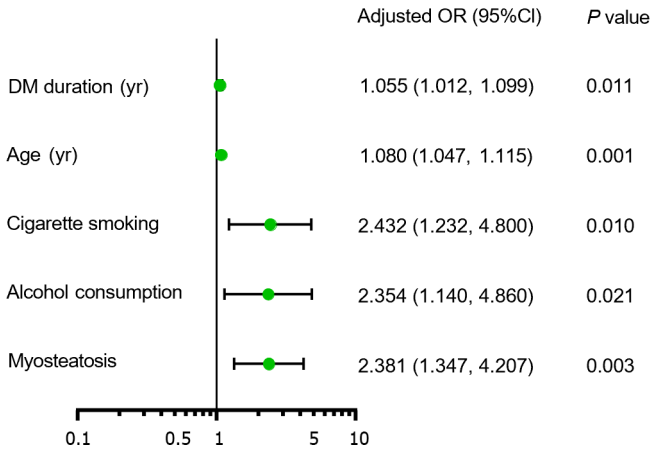

Figure 1 Forest plot of independent factors identified from logistic linear regression for severe coronary artery calcification. DM: Diabetes mellitus.

the cut-off age values for predicting SCAC to be > 56.5 years in males and > 63.5 years in females. This finding aligns with a previous study, which revealed that the prevalence of CACS > 0 exceeded 25% in young males with at least one risk factor by the age of 40, and in young females with at least one traditional risk factor by the age of 50[31].

Muscle mass has been regarded as a predictor for coronary atherosclerosis in previous studies[32,33]. However, these studies are limited by the use of dual-energy X-ray absorptiometry or bioelectrical impedance analysis, which are not allowed to be used to evaluate muscle quality. In our study, both logistic regression and CHAID decision tree analyses showed no significant association between SMI and SCAC, even when SMI was transferred into a binary variable according to the diagnostic criteria of low muscle mass (data not shown)[9]. This result is consistent with the study by Lee *et al*[4], which assessed the association between muscle quality and CAC in the general population. Therefore, myosteatosis might play a more important role than low muscle mass in the development of CAC, especially in the population with T2DM.

CHAID algorithm for decision tree analysis was used to visualize the relationship between SCAC and related factors in an easy-to-interpret tree image. Myosteatosis was a primary factor for SCAC in younger patients and was associated with a more than two-fold increased risk of SCAC. Therefore, the occurrence of severe atherosclerosis in certain younger individuals might be attributed to myosteatosis. It is important to note that while myosteatosis was not included in the CHAID decision tree analysis for the older age subgroup, the quality of muscle in elderly patients remains significant.

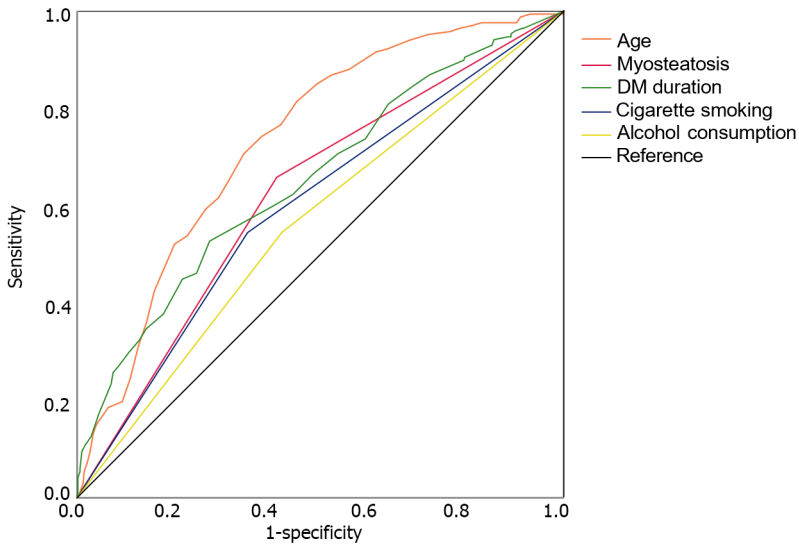

Figure 2 Receiver operating characteristic curve analysis of risk factors for severe coronary artery calcification alone or in combination. DM: Diabetes mellitus.

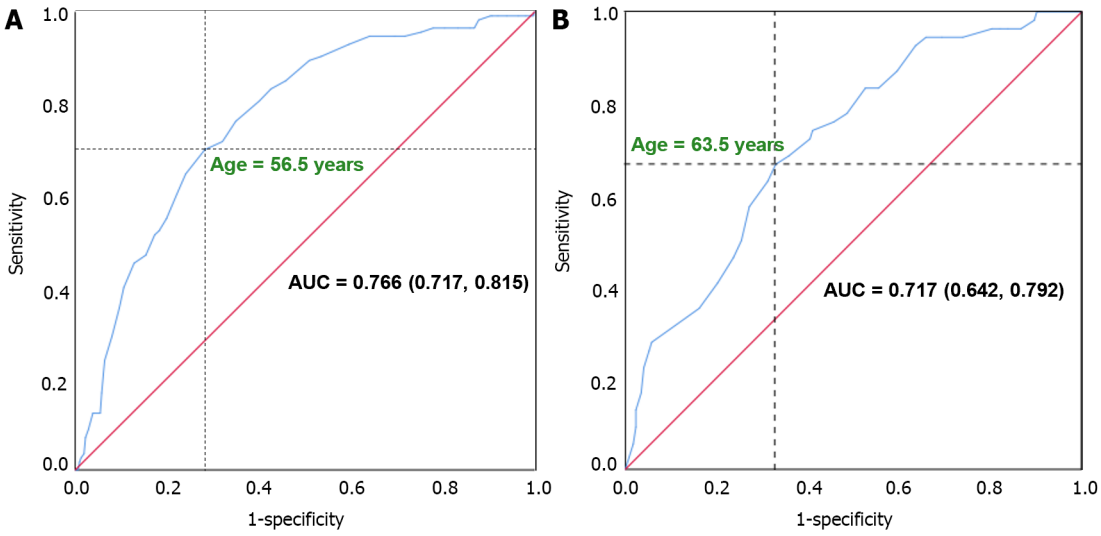

Figure 3 Cut-off points of age for severe coronary artery calcification. A. Subgroup of males; B. Subgroup of females. AUC: Area under the curve.

Our study revealed that approximately 65.8% of older patients were diagnosed with myosteatosis (data not shown), and therefore, myosteatosis cannot truly reflect the difference in their muscle quality. Thus, large epidemiological studies are needed to establish an improved criterion for myosteatosis based on age, especially for myosteatosis in elderly individuals.

In addition to CAC, we also assessed the associations of myosteatosis with diabetes complications, hormonal status, and medication usage. Although no difference was found in the risk of diabetes complications, patients with myosteatosis exhibited a higher risk of CHD. This finding supports our conclusion regarding the association between myosteatosis with SCAC. Hormonal status plays a crucial role in maintaining muscle health. In this cross-sectional study, patients with myosteatosis showed no significant differences in the levels of thyroid hormones. Further research is necessary to evaluate the association of myosteatosis with other hormones, including growth hormone, estrogen, testosterone, and adrenal hormones. Patients with myosteatosis had a higher prevalence of insulin, statins, and aspirin usage. However, this does not imply that these medications induce myosteatosis, as patients with myosteatosis require these medications due to their elevated risk of CHD and lower levels of HOMA2-β.

Our study has several limitations. First, the characteristics of the cross-sectional study limited the further exploration of the causal inference and the clarification of the underlying pathophysiological mechanism between myosteatosis and coronary atherosclerosis. Second, we did not assess the muscle function (*e.g.*, handgrip strength and gait speed), which is highly associated with muscle quality[34]. Third, some information that may be associated with CAC, such as the family history of premature cardiovascular disease and the physical activity of patients, was missing. Fourth, our study did not analyze the association of myosteatosis with the features of plaque vulnerability, such as volume and density, which may

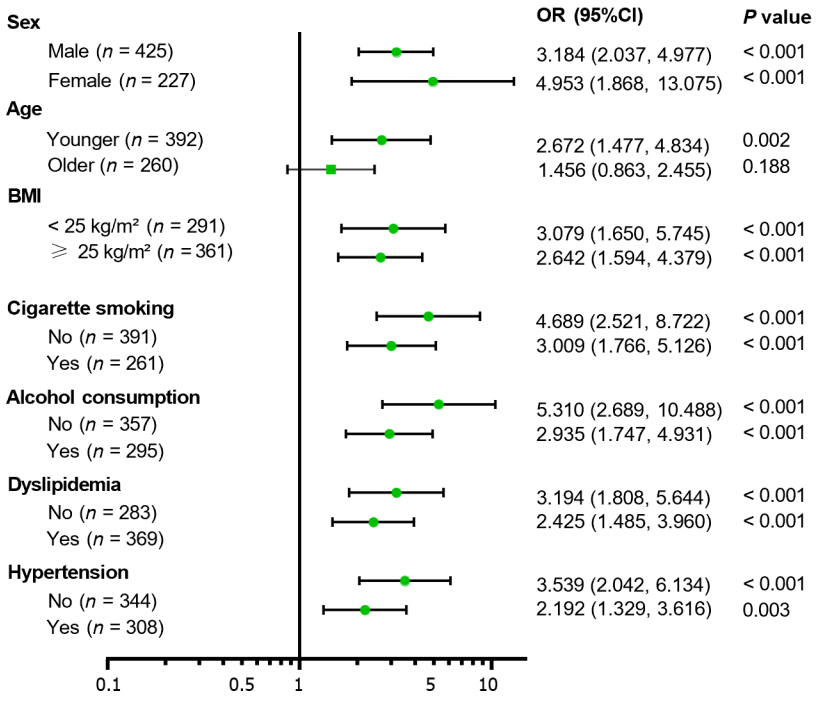

Figure 4 Subgroup analyses of myosteatosis in predicting severe coronary artery calcification. BMI: Body mass index.

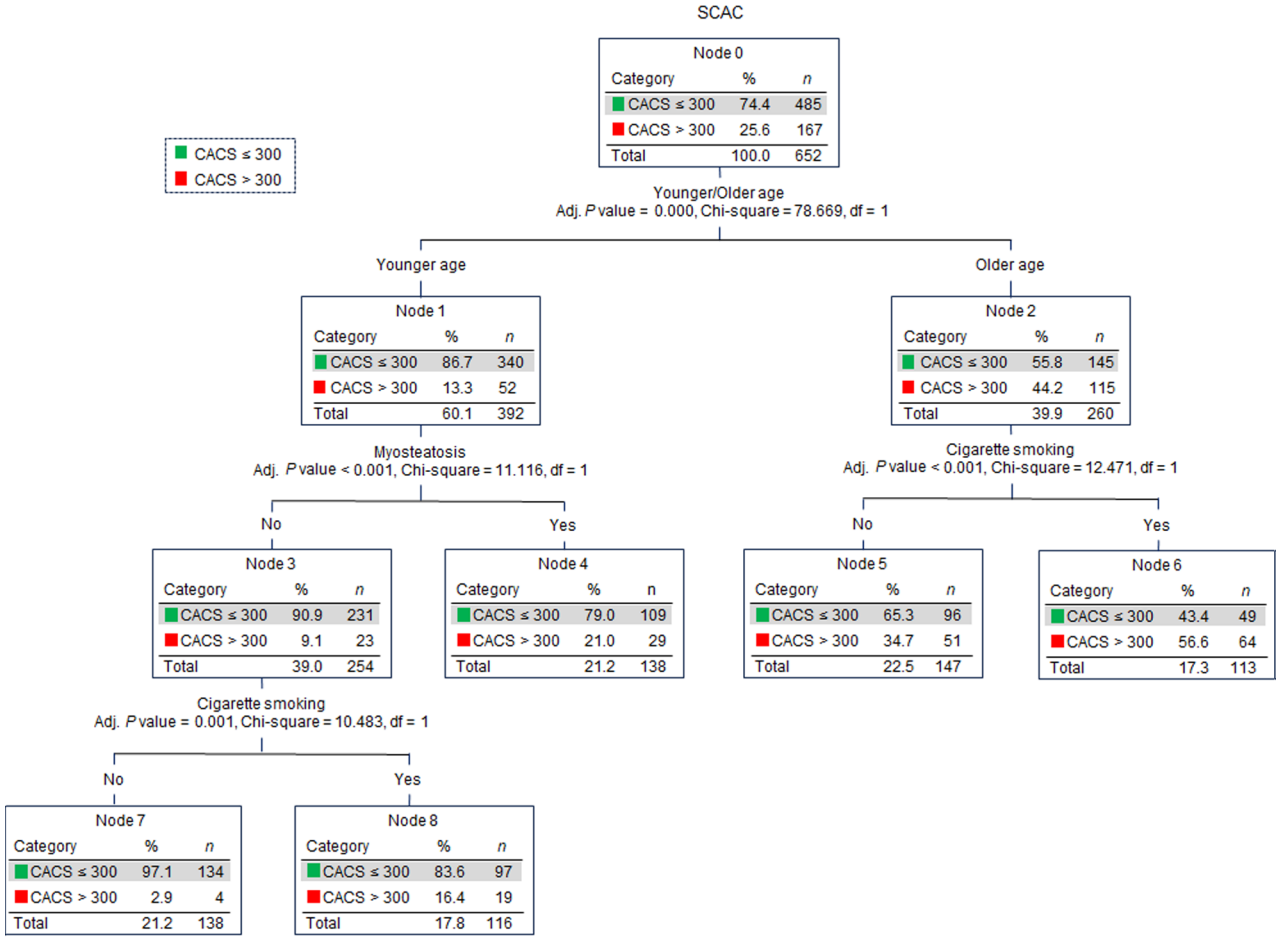

Figure 5 Chi-squared automatic interaction detection decision tree analysis. SCAC: severe coronary artery calcification; CACS: Severe coronary artery calcification score.

have opposite relationships with cardiovascular events[35]. Lastly, because our study was conducted in Chinese adults with T2DM, the findings may not be readily generalizable to other populations or ethnicities.

However, our study also has several strengths. First, this study included a large sample of 652 individuals and used CT-derived measures of both myosteatosis and SCAC. Second, our study focused on patients with T2DM and this population has a high prevalence of both myosteatosis and atherosclerotic cardiovascular diseases. Third, most of the important biochemical variables were available and all the diabetic complications were assessed by professional clinicians. Fourth, the CHAID decision tree analysis highlighted that the association between myosteatosis and SCAC might be more prominent in individuals with younger ages and lower risks of atherosclerosis. This is a novel finding of our study.

CONCLUSION

In conclusion, myosteatosis was a novel risk factor for atherosclerosis in patients with T2DM, especially in the population with younger ages or fewer traditional risk factors. This suggests the potential benefit of initiating muscle-strengthening exercises and improving muscle quality at a younger age. Further follow-up studies are warranted to validate the role of myosteatosis in cardiovascular events or mortality in patients with T2DM.

ARTICLE HIGHLIGHTS

Research background

Myosteatosis rather than low muscle mass is the major etiologic factor of sarcopenia in patients with type 2 diabetes mellitus (T2DM). Myosteatosis may lead to a series of metabolic dysfunctions which are closely associated with acceleration of T2DM and atherosclerosis.

Research motivation

The association between myosteatosis and coronary atherosclerosis in patients with T2DM has not been reported yet.

Research objectives

To investigate the association between myosteatosis and coronary artery calcification (CAC) in patients with T2DM.

Research methods

Severe CAC (SCAC) was defined when the CAC score was > 300. Logistic regression and decision tree analyses were performed to assess the association between myosteatosis and SCAC.

Research results

Myosteatosis was significantly associated with increased risk of SCAC. The association between myosteatosis and SCAC was significant in the younger, rather than older patients, and was more prominent in the population with lower risks of atherosclerosis.

In the patients with older age, the main factor for SCAC was cigarette smoking, while in the patients with younger age, the main factor was myosteatosis.

Research conclusions

Myosteatosis was a novel risk factor of atherosclerosis in patients with T2DM, especially in the population with younger age or lower traditional risk factors.

Research perspectives

Follow-up studies are warranted to confirm the role of myosteatosis in cardiovascular events or mortality in patients with T2DM.

FOOTNOTES

Co-corresponding authors: Yan-Gang Wang and Mei Zhang.

Author contributions: Zhang M and Wang YG designed research; Zhang M, Wang YG and Liu FP contributed to study protocols and analysis plans; Liu FP, Guo MJ, Yang Q, and Li YY interpreted the data; Liu FP and Yang Q drafted the manuscript; Liu FP, Yang Q, and Guo MJ were the guarantors of this work, thereby having full access to all the data in the study and taking responsibility for the data's integrity and accuracy of the analysis; all authors were involved in the critical review of the results and have contributed to, read, and approved the final manuscript. Zhang M and Wang YG were designated as co-corresponding authors. First, the research was performed as a collaborative effort, and the designation of co-corresponding authors accurately reflected the distribution of responsibilities and efforts involved in completing the study and the resulting paper. Second, the research team encompassed authors with a variety of

expertise and skills from different fields, making the designation of co-corresponding authors the most suitable choice to reflect this diversity. Third, Zhang M and Wang YG made substantial and equal contributions throughout the research process. Selecting these researchers as co-corresponding authors acknowledged and respected their equal contributions while recognizing the spirit of teamwork and collaboration in this study. In summary, we believe that designating Zhang M and Wang YG as co-corresponding authors is appropriate for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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ORIGINAL ARTICLE

Retrospective Study

Adherence to Advisory Committee on Immunization Practices in diabetes mellitus patients in Saudi Arabia: A multicenter retrospective study

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Abstract

BACKGROUND

Patients with diabetes mellitus (DM) are predisposed to an increased risk of infection signifying the importance of vaccination to protect against its potentially severe complications. The Centers for Disease Control and Prevention/Advisory Committee on Immunization Practices (CDC/ACIP) issued immunization recommendations to protect this patient population.

AIM

To assess the adherence of patients with DM to the CDC/ACIP immunization recommendations in Saudi Arabia and to identify the factors associated with the vaccine adherence rate.

METHODS

An observational retrospective study conducted in 2023 was used to collect data on the vaccination records from 13 diabetes care centers in Saudi Arabia with 1000 eligible patients in phase I with data collected through chart review and 709 patients in phase II through online survey.

RESULTS

Among participants, 10.01% (n = 71) had never received any vaccine, while 85.89% (n = 609) received at least one dose of the coronavirus disease 2019 (COVID-19) vaccine, and 34.83% (n = 247) had received the annual influenza vaccine. Only 2.96% (n = 21), 2.11% (n = 15), and 1.12% (n = 8) received herpes zoster, tetanus, diphtheria, and pertussis (Tdap), and human papillomavirus (HPV) vaccines, respectively. For patients with DM in Saudi Arabia, the rate of vaccination for annual influenza and COVID-19 vaccines was higher compared to other vaccinations such as herpes zoster, Tdap, pneumococcal, and HPV. Factors such as vaccine recommendations provided by family physicians or specialists, site of care, income level, DM-related hospitalization history, residency site, hemoglobin A1c (HbA1c) level, and health sector type can significantly influence the vaccination rate in patients with DM. Among non-vaccinated patients with DM, the most reported barriers were lack of knowledge and fear of side effects. This signifies the need for large-scale research in this area to identify additional factors that might facilitate adherence to CDC/ACIP vaccine recommendations in patients with DM.

CONCLUSION

In Saudi Arabia, patients with DM showed higher vaccination rates for annual influenza and COVID-19 vaccines compared to other vaccinations such as herpes zoster, Tdap, pneumococcal, and HPV. Factors such as vaccine recommendations provided by family physicians or specialists, the site of care, income level, DM-related hospitalization history, residency site, HbA1c level, and health sector type can significantly influence the vaccination rate in patients with DM.

Key Words: Diabetes mellitus; Vaccine recommendation; COVID-19 vaccine; Influenza vaccine; Pneumococcal vaccine; Immunization; Retrospective study

Core Tip: Given the increasing prevalence of diabetes in Saudi Arabia, this national study sheds light on vaccine practices for patients with diabetes mellitus in Saudi Arabia with regard to the Advisory Committee on Immunization Practices vaccine recommendations. The findings of this protocol will aid decision-makers in improving preventative vaccine care for patients with diabetes.

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INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder characterized by long-term elevated blood sugar levels due to insufficient insulin production, insulin resistance, or both[1]. Globally, data from the International Diabetes Federation reveals that in 2021, approximately 537 million adults worldwide were affected by DM. Projections suggest that this number is expected to increase to 643 million by 2030 and further escalate to 783 million by 2045[2]. In Saudi Arabia, DM impacts roughly 20% of the adult population, with projections indicating that by 2030, the number of cases will more than double[3].

Worldwide, the burden of DM has led to approximate health expenses of 966 billion United States dollars. These expenses are projected to surpass 1.054 trillion United States dollars by 2045[4]. In Saudi Arabia, the escalating prevalence of DM is emerging as a significant contributor to medical complications and fatalities, imposing an economic burden measured at 17 billion Saudi riyals in 2018[5].

Vaccinations play a crucial role in preventing infectious diseases and promoting immunity, particularly for individuals with DM. This significance is evident in the 2011-2020 Global Vaccine Action Plan, built on the ideal that "The benefits of immunization to be equitably extended to all people"[6]. This includes high-risk groups vulnerable to vaccine-preventable diseases, such as patients with chronic and immune-compromising diseases[7]. Factors like impaired immunity, a prolonged course of the disease, poor diabetes control, hyperglycemia, and comorbidities make patients with DM more susceptible to infections and serious complications[8].

According to the World Health Organization, the mortality rate in cases of pneumococcal infection is estimated to be approximately 10%-20%, with rates exceeding 50% in high-risk populations. It is assumed that patients with DM who develop pneumonia-related complications face a nearly threefold higher risk of mortality in comparison to the general population[9]. Annually, influenza is responsible for approximately 10000 to 30000 fatalities, and individuals with DM have a sixfold increased likelihood of hospitalization during an outbreak compared to those without DM[8]. Through extensive efforts to promote vaccination within this vulnerable population, which have shown promising results, a study indicated that the influenza vaccine effectively reduced rates of hospitalization and mortality, with a number needed to treat of 60, 319, and 250 for all-cause hospitalizations, specific hospitalization, and all-cause mortality, respectively[10]. Another study demonstrated a decline in the risk of invasive pneumococcal disease (adjusted odds ratio = 0.86, 95% confidence interval: 0.78-0.94) among vaccinated patients compared to unvaccinated patients, along with a shorter length of stay at the hospital (-1.27 ± 0.19 d, P = 0.0012)[11]. This reinforces the importance of implementing vaccine recommendations and strictly encouraging adherence to these vaccinations.

Similar to other adults and as recommended by the Centers for Disease Control and Prevention (CDC) Advisory Committee on Immunization Practices (ACIP), patients with DM should receive vaccinations against coronavirus disease 2019 (COVID-19), influenza, tetanus, diphtheria, and pertussis (Tdap), as well as tetanus and diphtheria boosters. Additionally, individuals with DM must also receive the pneumococcal vaccine, hepatitis B vaccine, human papillomavirus (HPV) vaccine, herpes zoster (Shingles) vaccine, measles, mumps, and rubella (MMR) vaccine, and chickenpox vaccine. The CDC/ACIP recommendations are summarized in Table 1[12].

Worldwide, adherence to these vaccinations among adult patients with DM has been investigated in a few single-center studies. A cross-sectional study conducted at Kent Hospital in the United Kingdom, involving 100 patients, revealed a notably low adherence rate to the ACIP recommendations for hepatitis B, pneumococcal, and influenza vaccines among patients with DM. Specifically, for the hepatitis B vaccine, 39% of vaccine-eligible patients reported non-compliance[13]. Additionally, a recent meta-analysis study noted that 27.8% of patients expressed reluctance to receive the COVID-19 vaccination[14].

To date, only three studies have been conducted in Saudi Arabia to assess vaccination adherence among patients with DM. These studies were either single-centered or focused on only one to three vaccines. A study conducted in Taif City among 336 patients found that only 43.5% of patients with DM received the influenza vaccine, contradicting the 61% expected adherence rate reported in 2017[15]. Another study at King Abdulaziz University Hospital in Jeddah showed a very low uptake rate of influenza, pneumococcal, and hepatitis B vaccines among admitted patients with DM, with only 1.17% of the 832 participants receiving all three vaccines[16].

MATERIALS AND METHODS

This study was conducted over two phases. Phase I included an observational retrospective chart review from 13 diabetes care centers in Saudi Arabia. Subsequently, in phase II, we administered an online survey with electronic consent to patients with DM who have established care at those 13 diabetes care centers, aiming to gather additional information on socioeconomic, educational, and living status.

Participants' vaccine records were reviewed by two independent teams of physicians and assessed for adherence to the latest vaccine recommendations announced in 2023 by ACIP and the CDC. Demographic data were collected from each participant, including gender, age, education, monthly household income, and living status, which was divided into two categories: City (a large human settlement with a significant population and extensive facilities) and village (a smaller settlement situated in a rural area with a small population ranging from hundreds to thousands), as well as body weight. Additionally, we collected data on hemoglobin A1c (HbA1c) levels, pre-existing conditions, DM duration, healthcare sector (either governmental sector - a public sector that provides free health-related services for Saudi citizens - or private sector - centers that deliver health services for all residents of the country and are funded by self-pay or insurance), preference for diabetic care (whether primary health care centers - centers provided by the Ministry of Health to offer primary health care to the regions it serves through applying a comprehensive care strategy for family medicine - or diabetes care centers - a center with a specialized diabetologist or endocrinologist; or none), frequency of diabetes provider visits (monthly, quarterly, annually, or none), diabetes regimen (oral medications, insulin, insulin and oral medications, no medications), frequency of total daily medications, and previous hospitalization due to diabetes complications. Finally, data about the reasons for non-adherence to vaccinations among non-vaccinated patients with DM were collected, with patients choosing one of several reasons: "I do not know the importance of these vaccines for diabetes", "fear of side effects", "the vaccines were not suggested by the doctor", "I think the vaccine is not important", "not educated about the importance of vaccines by the doctor", "lack of vaccine", or "reason not disclosed".

We expressed categorical variables as frequencies and percentages, while continuous variables were presented as means and standard deviations or as medians and minimum-maximum ranges. To compare continuous variables between two groups, we utilized an independent Student's *t*-test, while a one-way ANOVA was employed for the comparison of more than two groups. Additionally, we utilized the Tukey test for multiple comparisons of the subgroups. A predetermined significance level of $P < 0.05$ was used to detect differences between study groups. The statistical analysis was conducted using the SPSS version 26.0. Bar diagrams were generated using GraphPad Prism version 9.0. The study protocol (607-43-6007) received IRB approval from the Regional Research Ethics Committee, Ministry of Health, Saudi Arabia.

Table 1 Centers for Disease Control and Prevention/Advisory Committee on Immunization Practices vaccine recommendations for diabetic patients

Vaccine	Recommendation
IIV (IIV4 or RIV4 or LAIV)	Received every year
Tdap and Td vaccine	Tdap is received once followed by a Td booster dose every ten years
PCV (PCV15[1] or PCV20)	Given once to previously unimmunized diabetic adults who are 19-64 years old or unimmunized adults ≥ 65
Hepatitis B vaccine	All previously unimmunized adults 19-59 years old. Diabetic adults ≥ 60 years old
HPV vaccine	Given in two or three doses as early as 9 years old and up to 26 years old and in some cases up to 47 years old
Herpes zoster vaccine	All adults ≥ 50
Chickenpox (varicella) vaccine	Two doses with 4-8 wk interval to all previously unimmunized ≥ 13 adolescents and adult
MMR vaccine	One or two doses with 28 d interval for unimmunized adults

[1]Pneumococcal vaccine 15 is followed by a 23-valent pneumococcal polysaccharide vaccine booster at least 1 year later.
IIV: Influenza vaccine; RIV: Recombinant influenza vaccine; LAIV: Live-attenuated influenza virus; Tdap: Tetanus, diphtheria, and pertussis; Td: Tetanus and diphtheria; PCV: Pneumococcal vaccine; HPV: Human papillomavirus; MMR: Measles, mumps, and rubella.

RESULTS

Baseline characters

Out of 1000 eligible patients whose charts were reviewed in phase I, a total of 709 adult patients with DM consented and participated in phase II, being included in this study. Among the 709 adults with DM surveyed, the majority were between 46 and 55 years old, with 55.7% of participants being female. Most patients were educated, with 55.9% having a bachelor's degree. The majority of participants in our study had a long-standing disease of more than 10 years (42.5%). Baseline characteristics are depicted in Table 2.

Rate of vaccinations among study participants

Figure 1 illustrates the varied vaccination rates among the study participants. It is notable that a small minority, 10.01% (n = 71), have never been administered any form of vaccine. Conversely, a substantial majority, 85.89% (n = 609), have received at least one dose of the COVID-19 vaccine. Additionally, 34.83% (n = 247) of participants had been administered the annual influenza vaccine. However, the reception for other vaccines was notably lower, with only 2.96% (n = 21) having received the herpes zoster vaccine, 2.11% (n = 15) the Tdap vaccine, and a mere 1.12% (n = 8) being administered the HPV vaccine.

Impact of care site on vaccination rates

A one-way ANOVA was conducted to determine if there is a relationship between the site of care and the frequency of vaccines received by patients. Patients were classified into three groups according to their care site: Primary care center, provided by the Ministry of Health to offer primary health care to the region it serves, applying a comprehensive care strategy for family medicine (n = 256), diabetes center (n = 296), and no designated center of care (n = 157). One-way ANOVA indicated the presence of a statistically significant difference between sites of care. Post-hoc Tukey HSD test revealed that when compared to the patients who receive care from the diabetes center, the patients who have no designated center for DM care had a significantly lower mean frequency of vaccines received (mean different = 0.23, P = 0.015). In addition, no significant difference was found between the other groups (Figure 2).

Income-level disparities in vaccine uptake

A one-way ANOVA was conducted to determine if there is a relationship between the frequency of vaccines received and different income levels. Patients were classified into five groups according to their income level: Not disclosed (n = 265), > 9000 Saudi Arabian Riyal (SAR) (n = 145), 6000-9000 SAR (n = 67), 4500-5999 SAR (n = 186), < 4500 SAR (n = 46). One-way ANOVA indicated the presence of a statistically significant difference between the income groups. Post-hoc Tukey HSD test revealed that, when compared to the > 9000 SAR income group, the 4500-5999 SAR and the 6000-9000 SAR income groups had a significantly lower mean frequency of vaccines received (mean different = 0.25, P = 0.042, and mean different = 0.38, P = 0.014) respectively. In addition, no significant difference was found between the other groups (Figure 3).

Vaccination uptake across various educational levels

A one-way ANOVA was conducted to determine if there is a relationship between the frequency of vaccines received and the educational level of the patients. Patients were classified into four groups according to their education level: Less than primary school (n = 36), school (n = 225), graduate (n = 396), and postgraduate (n = 52). One-way ANOVA indicated a

Table 2 Baseline characteristics of study participants

	Overall (*n* = 709)
Age (yr)	
18-25	172 (24.3%)
26-35	82 (11.6%)
36-45	115 (16.2%)
46-55	177 (25.0%)
56-65	123 (17.3%)
Above 65	40 (5.6%)
Gender	
Male	314 (44.3%)
Female	395 (55.7%)
HbA1C	
Less than 7%	202 (28.5%)
From 7%-8%	226 (31.9%)
From 8%-10%	200 (28.2%)
More than 10	81 (11.4%)
Weight, mean ± SD	76.72 ± 19.5
Pre-existing conditions	
Heart disease	101 (14.24%)
Hypertension	271 (38.22%)
Dyslipidemia	241 (33.99%)
Thyroid disease	82 (11.56%)
None	233 (32.9%)
Education level	
Below primary school	36 (5.1%)
School (public education)	225 (31.73%)
Bachelor's degree	396 (55.9%)
Post graduate degree	52 (7.3%)
Living status	
City	594 (83.8%)
Village	115 (16.2%)
Monthly household income	
< 4500 SAR	46 (6.5%)
4500-5999 SAR	186 (26.23%)
6000-9000 SAR	67 (9.44%)
> 9000 SAR	145 (20.45%)
Not disclosed	265 (37.37%)
Diabetes duration (yr)	
< 1	89 (12.6%)
1-5	185 (26.1%)
6-10	134 (18.9%)
> 10	301 (42.5%)

Health care sector	
Government	547 (77.2%)
Private	162 (22.8%)
Preference for diabetes care	
Primary care centers	256 (36.1%)
Diabetes care centers	296 (41.7%)
None	157 (22.1%)
Frequency of diabetes provider visits	
Monthly	263 (37.1%)
Quarterly	230 (32.4%)
Annually	207 (29.2%)
None	9 (1.3%)
Diabetes regimen	
Oral mediations	246 (34.7%)
Insulin	299 (42.17%)
Insulin and oral medications	91 (12.8%)
No medications	73 (10.29%)
Number of total daily medications	
0-2 medications	333 (47%)
3-4 medications	235 (33.1%)
5-9 medications	129 (18.2%)
10 medications	12 (1.7%)
Previous hospitalization due to diabetes complication	
Heart attack	20 (2.82%)
Diabetic foot	27 (3.8%)
Pneumonia	74 (10.43%)
Numbness in the limbs	288 (40.62%)
Kidney disease	26 (3.66%)
Stroke	24 (3.38%)
Diabetes related vision problems	116 (16.36%)
Hepatitis	10 (1.41%)
Shingles	7 (0.98%)
Erectile dysfunction	20 (2.82%)
Previous depression diagnosis	138 (19.46%)

HbA1C: Hemoglobin A1c; SAR: Saudi Arabian Riyal.

non-significant difference between the different education groups with a *P*-value of 0.233 (Figure 4).

Comparison of vaccination rates between hospitalized and non-hospitalized patients with diabetes

An independent *t*-test was performed to compare the frequency of vaccines received by diabetic patients with a history of hospitalization to those without. As seen in Figure 5, the analysis concluded that the group with a hospitalization history (mean = 1.42, SD = 0.83) received a significantly higher frequency of vaccinations [$t(707) = 3.10$, $P = 0.002$] compared to the non-hospitalized group (mean = 1.20, SD = 0.78).

Comparative analysis of vaccination rates between city and village residents

We performed an independent *t*-test to compare the frequency of vaccines received between city residents and village

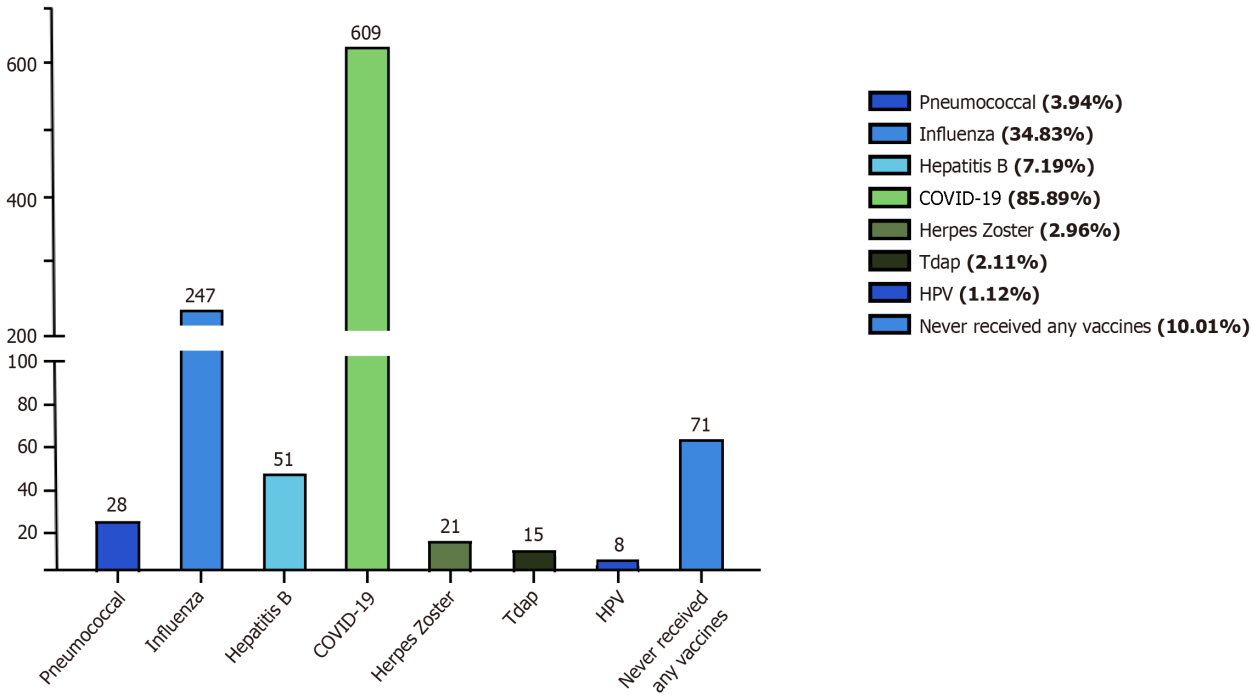

Figure 1 Vaccination rates among patients with diabetes mellitus. COVID-19: Coronavirus disease 2019; HPV: Human papillomavirus.

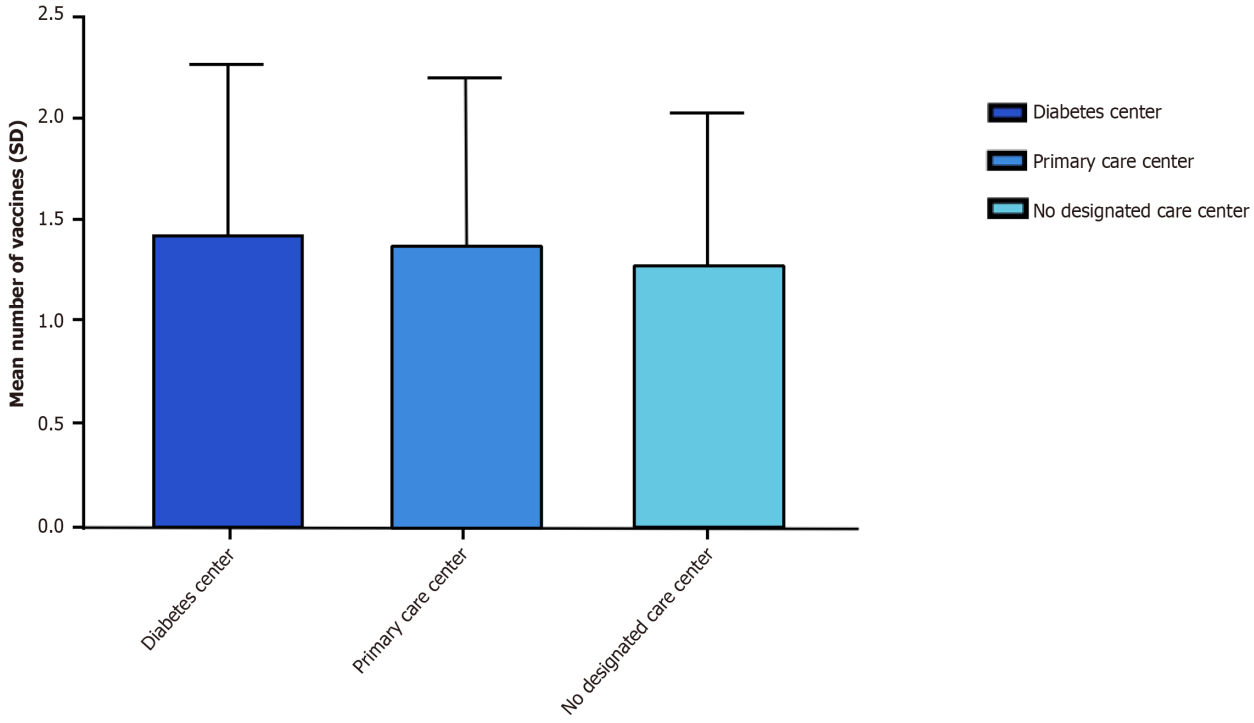

Figure 2 The relationship between the site of care and the frequency of vaccines received by patients.

residents. The test concluded that village residents (mean = 1.53, SD = 0.93) had received a significantly higher frequency of vaccinations [$t(707) = 2.33$, $P = 0.02$] than city residents (mean = 1.33, SD = 0.79) (Figure 6).

Comparison of vaccination adherence between patients in government and private healthcare sectors

We performed an independent *t*-test to compare the frequency of vaccines received between the patients receiving care from government hospitals and the patients receiving care from private hospitals (Figure 7). The results concluded that patients in government sectors (mean = 1.40, SD = 0.82) had significantly higher adherence to vaccinations [$t(707) = 2.22$, $P = 0.02$] than patients in private sectors (mean = 1.24, SD = 0.80).

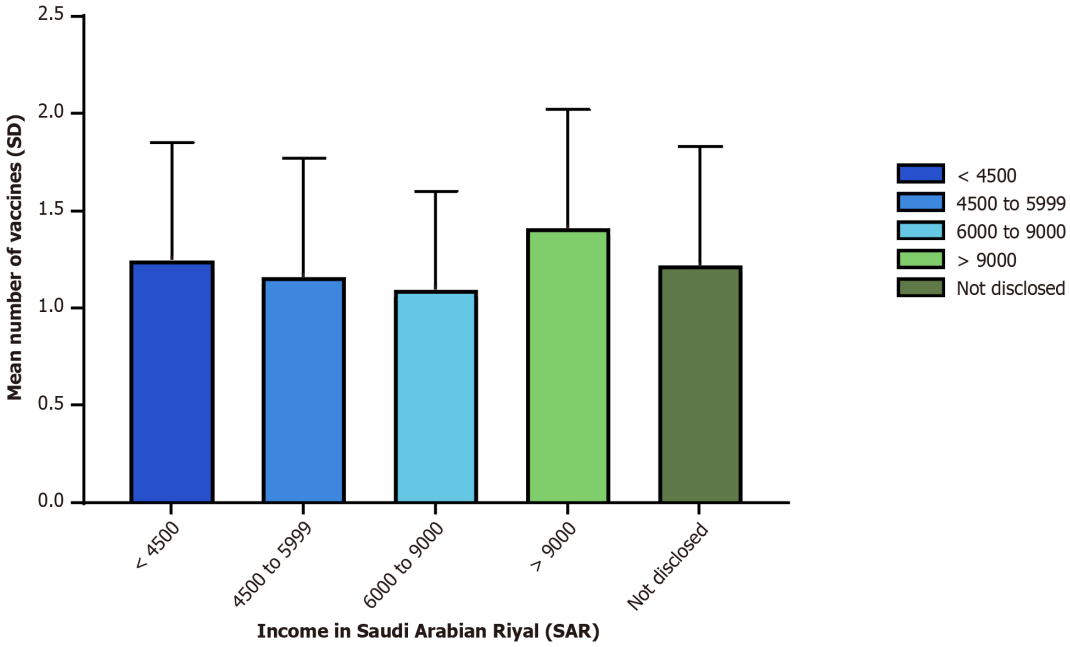

Figure 3 Comparison of vaccine uptake among different income levels. SAR: Saudi Arabian Riyal.

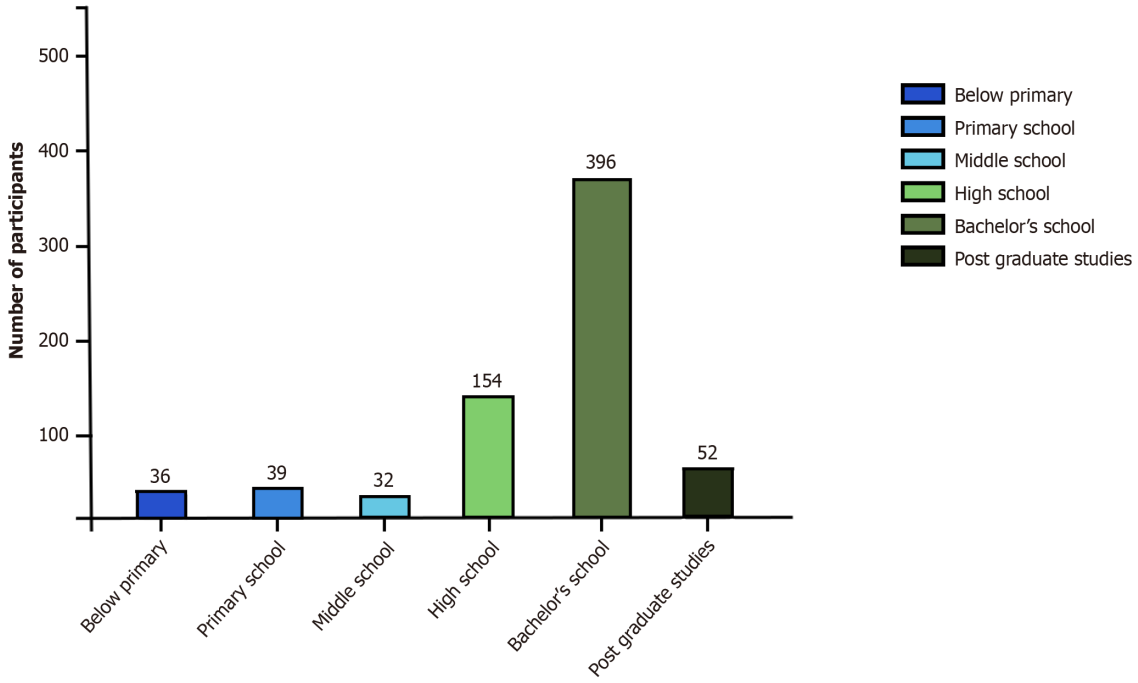

Figure 4 A comparative analysis of vaccination rates across varying educational attainment levels.

Comparative analysis of vaccination frequency in relation to glycemic control levels

We performed an independent *t*-test to compare the frequency of vaccines received between those with HbA1c < 8% and those with HbA1c > 8% (Figure 8). The results concluded that patients with HbA1c > 8% (mean = 1.41, SD = 0.86) had received a significantly higher frequency of vaccinations [$t(707) = 2.14$, $P = 0.03$] than patients with HbA1c < 8% (mean = 1.26, SD = 0.71).

Barriers to vaccine adherence in non-vaccinated patients with DM

Our survey has shown that 71 out of 709 participants did not receive any vaccine. Table 3 demonstrates the most commonly reported barriers to receiving vaccination by patients with DM. The most prevalent reasons were lack of knowledge about the vaccines' importance and fear of side effects, reported by 29.57% ($n = 21$) and 28.16% ($n = 20$), respectively. On the other hand, lack of vaccines was the least commonly disclosed barrier, reported by only 2.81% ($n = 2$).

Table 3 Barriers to vaccination adherence reported by non-vaccinated patients with diabetes mellitus

Barrier	Participants, *n* (%)
I do not know the importance of these vaccines for diabetes	21 (29.57)
Fear of side effects	20 (28.16)
The vaccines were not suggested by the doctor	11 (15.49)
I think the vaccine is not important	10 (14.08)
Not educated about the importance of vaccines by the doctor	6 (8.45)
Lack of vaccine	2 (2.81)
Reason not disclosed	4 (5.63)

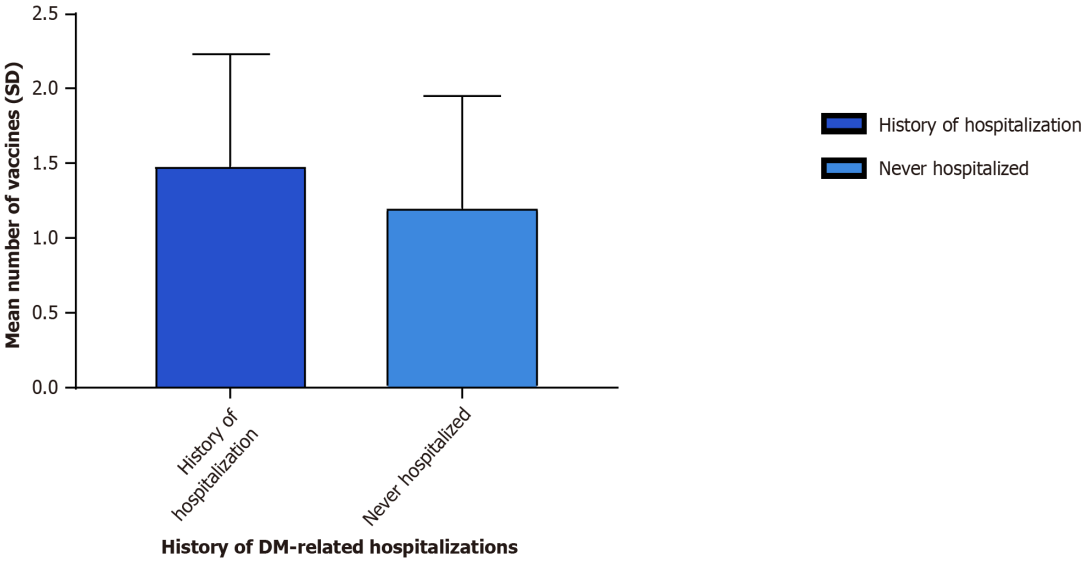

Figure 5 Comparison of vaccination rates between previously hospitalized patients with diabetes and those with no hospitalization history. DM: Diabetes mellitus.

DISCUSSION

The findings from 709 patients with DM in Saudi Arabia showed that 34.83% of participants received the annual influenza vaccine, and 85.89% received at least one dose of the COVID-19 vaccination. However, there is generally a low rate of other vaccinations, including herpes zoster, Tdap, pneumococcal, and HPV vaccines. In Saudi Arabia, MMR and varicella (chickenpox) vaccinations are included in the Saudi national vaccine schedule and are required for enrollment in the public education system[17].

Vaccine recommendations delivered to patients, either from their family physicians or specialists, can have an impact on vaccination acceptance by the patient[18]. In our study, we showed that participants without a designated care center exhibited significantly lower mean frequency of vaccination rates when compared to those who received care from a diabetes center. This finding is consistent with another study that showed higher vaccination coverage among patients reporting frequent physician visits[19]. Interestingly, the vaccination rates when comparing primary health care centers and specialized diabetic centers were not significantly different in our report. This contrasts with another report that considered visits to specialists as an independent factor in pneumococcal vaccination compared to family doctors[20]. Additionally, another study reported that patients with DM expressed more trust and willingness to take vaccines when advised by their diabetologist compared to family physicians, at rates of 80.9% and 50.9%, respectively[21].

The impact of socioeconomic status on vaccination rates has been investigated in previous studies. Research conducted in the United States, Thailand, and South Korea has shown that socioeconomic factors are related to unvaccinated status, especially among vulnerable groups such as young adults, individuals without insurance, low-income families, and those lacking access to medical care[22-24]. Our findings align with these studies, highlighting a significantly positive correlation between income levels and vaccination status. In our study, higher income emerged as an important factor associated with the likelihood of being vaccinated.

Regarding education level, findings from previous studies have been conflicting, particularly in relation to the association between education level and vaccination status. A study conducted in South Korea reported that individuals with higher education levels had lower vaccination rates[23]. In our study, we observed a non-statistically significant difference in vaccination status among individuals with varying educational levels, ranging from those below primary

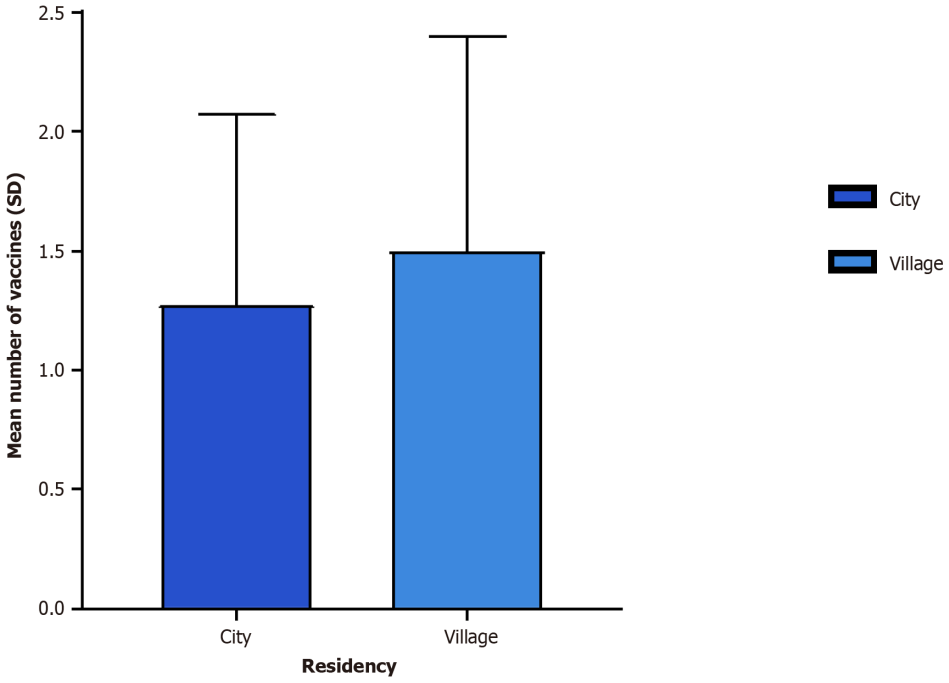

Figure 6 A comparative average frequency of vaccinations received by city and village residents.

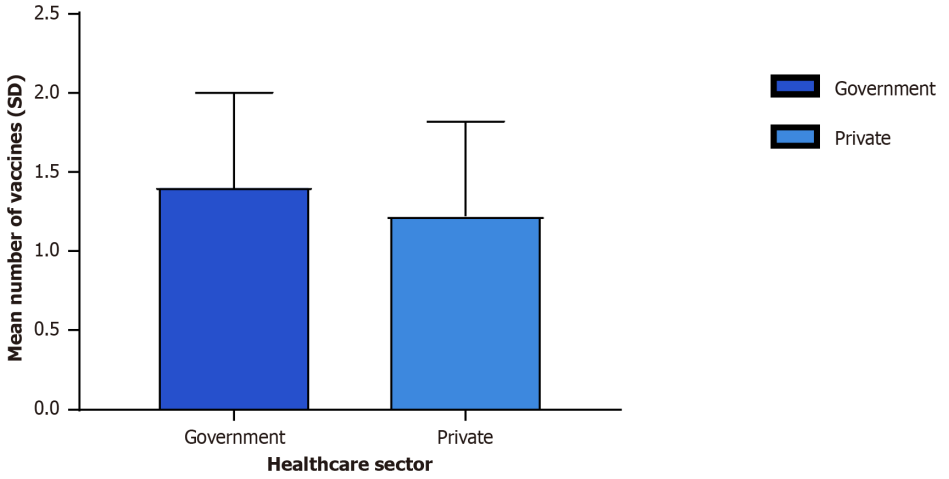

Figure 7 The difference in vaccination adherence between patients receiving care from government hospitals and those receiving care from private hospitals.

school to postgraduates. On the contrary, multiple previous studies have reported a significant positive correlation between higher educational levels and increased vaccination rates. For instance, a study conducted in Turkey aimed at determining vaccine awareness among patients with DM found a significant positive correlation between influenza vaccine acceptance and education level, suggesting that a higher education level increases the likelihood of accepting the vaccine[25]. Similarly, a study in Turkey also determined that receiving pneumococcal and influenza vaccinations is associated with higher education levels in patients with DM[26]. Additionally, previous studies in Austria, the United States, and Poland reported that individuals with high educational levels show an increase in vaccination coverage[24,27,28]. However, other studies conducted in Italy, China, and Spain reported that low vaccination rates were correlated with high educational levels[24,29,30].

In our current analysis, one of our interests was to investigate whether a previous history of hospitalization could affect the decision of DM patients to receive the CDC/ACIP-recommended vaccines. Our data indicated a significantly higher uptake of vaccines among patients with DM who have a history of hospitalization compared to those who have never been hospitalized due to DM complications. The results presented in a study by Lohan *et al*[31] provide a possible explanation for this finding. The study found an increase in vaccine coverage for influenza, Tdap, and pneumococcal vaccines in patients with DM after being admitted to an endocrinology department. Adherence to vaccines was especially noted in the department units that had an inpatient clinical pharmacist involved. This could be attributed to the fact that clinical pharmacists are more attentive to the patient's medication report and possess skills in educating patients about

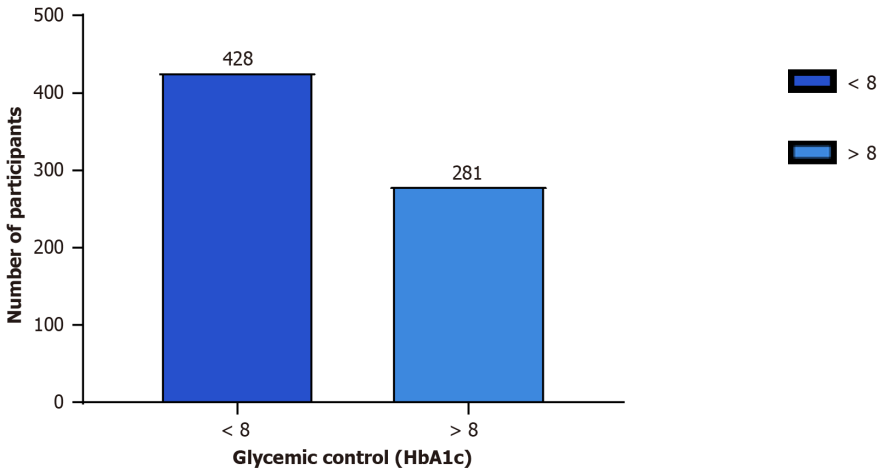

Figure 8 A comparative frequency of vaccinations received by patients with varying levels of glycemic control, measured by hemoglobin A1c. HbA1C: Hemoglobin A1c.

the importance of vaccinations, thereby facilitating higher vaccine compliance[31]. This privilege of direct access to physicians, clinical pharmacists, and nurses who can provide information about the vaccines and address the patient's concerns may explain the increased rate of vaccination in patients with a history of DM-related hospitalizations. Additionally, our study's findings align with the results of the study of Hung *et al*[32], which demonstrated an increase in influenza vaccine uptake in patients with DM who reported being hospitalized during the preceding year.

One might assume that patients residing in urban areas, such as cities, would be more likely to get vaccinated for several reasons, including high accessibility to healthcare services, the abundance of vaccine promotion campaigns, increased awareness about infection risks, and the perceived effectiveness of vaccines. However, studies examining the relationship between residency (urban *vs* rural areas) and vaccine adherence have reported inconsistent findings. A cross-sectional study conducted in China investigated the rate of COVID-19 vaccination among hospitalized patients with DM and found that individuals living in rural areas were significantly less likely to be vaccinated with the COVID-19 vaccine [33]. Another study, which included two million patients with chronic diseases, including DM, documented that patients living in rural areas had significantly higher pneumococcal vaccination rates but lower influenza vaccination rates[34]. Conversely, our results showed a significantly higher adherence rate to vaccinations among village residents compared toxicity residents. This could be explained by the Ministry of Health's efforts in rural areas and the periodic vaccination campaigns sent to villages. Additionally, the close connection among people living in village communities could facilitate the spread of vaccination awareness among them. Our results align with findings from a study that evaluated the uptake rate of the pneumococcal vaccine in the United Kingdom among two million at-risk patients, showing higher vaccination rates in patients living in rural areas[35]. An additional large-scale study in China found a higher hesitancy rate for COVID-19 vaccination in residents of rural areas[36].

The impact of governmental and private healthcare sectors on vaccine coverage among patients with DM is an interesting area to investigate due to the lack of research in this domain. Our results revealed that patients with DM who were followed up in governmental centers received more vaccines compared to those seeking healthcare in private centers. This difference might be explained by financial reasons, as vaccines are provided for free in governmental centers, whereas the cost of vaccines is either covered by patients' own funds or through insurance claims in private centers.

Poor glycemic control increases the likelihood of infection-related morbidity and mortality in patients with DM; thus, vaccination is critical for this population. In our study, patients with poor glycemic control unexpectedly had higher vaccination rates compared to patients with better glycemic control. This could be because healthcare providers may prioritize vaccination for patients with poor glycemic control. Conversely, another study conducted in South Korea revealed that better glycemic control, evidenced by lower HbA1c levels, was associated with higher vaccine coverage. This was rationalized as poor glycemic control correlating with less adherence to medical advice and, therefore, lower vaccine coverage[23].

The adherence of patients diagnosed with DM to the recommended vaccinations is influenced by their attitudes and perceptions, which are shaped by personal beliefs and guidance from healthcare providers. In our analysis, we identified knowledge insufficiency and concerns regarding the potential side effects of vaccinations as the most prevalent barriers among non-vaccinated patients with DM. This observation aligns with findings from a study conducted in Spain, where fear of adverse events was reported as the most prevalent cause of non-adherence to the influenza vaccine among females with DM[37]. Additional reported barriers include misconceptions about the vaccines' efficacy in preventing infectious diseases and their complications, needle aversion, concerns about vaccination costs, and issues related to vaccine availability[38,39]. In our current study, only 2.81% of the participants justified missing their vaccine due to the shortage of vaccine supply at the centers.

The crucial role of healthcare providers, including physicians, nurses, and pharmacists, in shaping the vaccination attitudes and perceptions of patients with DM is notable. This was evident in the study by Lewis-Parmar and McCann [40], which highlighted a pronounced fourteen-fold increase in the vaccination uptake rate among patients with DM

following the delivery of vaccination recommendations by a healthcare provider[40]. Barriers hindering the effectiveness of healthcare providers' role in motivating the adherence of patients with DM to recommended vaccinations include inadequate knowledge about these vaccines and limited participation by diabetologists and endocrinologists in guiding patient attitudes toward vaccines[41].

This calls for several key recommendations, including the utilization of various communication mediums such as social media and awareness campaigns to effectively correct any misconceptions. Furthermore, integrating a reminder system into electronic medical records can aid healthcare providers in educating and encouraging patients with DM to take their recommended vaccinations. Additionally, implementing the Standing Order Protocol, which allows non-physician medical providers to assess the patient's eligibility for vaccines and administer them without a physician's order, can be an effective strategy.

CONCLUSION

In Saudi Arabia, patients with DM showed higher vaccination rates for annual influenza and COVID-19 vaccines compared to other vaccinations such as herpes zoster, Tdap, pneumococcal, and HPV. Factors such as vaccine recommendations provided by family physicians or specialists, the site of care, income level, DM-related hospitalization history, residency site, HbA1c level, and health sector type can significantly influence the vaccination rate in patients with DM. This signifies the need for large-scale research in this area to identify additional factors that might facilitate adherence to CDC/ACIP vaccine recommendations in patients with DM.

ARTICLE HIGHLIGHTS

Research background

Diabetes constitutes a major risk factor for all types of infection due to deficiency in immune system. Those infections are not only frequent, but also have more risk of progression into severe presentation and poorer response to treatment. Enhancing immunity through vaccinations helps protect against potentially severe complications of such infections. The Centers for Disease Control and Prevention/Advisory Committee on Immunization Practices (CDC/ACIP) issued immunization recommendations to protect this patient population.

Research motivation

Data on adherence to immunization recommendations in patients with diabetes mellitus (DM) in Saudi Arabia is scarce. Shedding some light on immunization practices in this patient group should aid healthcare providers and decision-makers in optimizing DM preventative care in Saudi Arabia.

Research objectives

This retrospective multicenter study objectives include assessing the adherence of patients with DM to the CDC/ACIP immunization recommendations in Saudi Arabia and identifying the factors associated with the vaccine adherence rate.

Research methods

This is a retrospective study conducted in two phases to collect data regarding immunization rate of diabetic patients in Saudi Arabia. Data from 1000 eligible patient were gathered in phase I through chart review from 13 diabetes care centers. In phase II of the study, 709 out of the 1000 patients were enrolled through answering an online survey.

Research results

After data analysis, 10.01% (n = 71) of participants had never received any vaccine. The number of vaccinated diabetic patient with coronavirus disease 2019 (COVID-19) vaccine was 85.89% (n = 609), and annual influenza, 34.83% (n = 247), which is higher compared to other vaccinations. Multiple factors were significantly related to the rate of vaccinations among patients with diabetes including site of care, income level, DM-related hospitalization history, residency site, hemoglobin A1c (HbA1c) level, and health sector type. Lacking enough knowledge regarding the importance of immunizations and concerns regarding vaccine side effects were major barriers for receiving vaccines. This highlights the importance of conducting larger studies to explore other risk factors that may encourage adherence to CDC/ACIP vaccine recommendations.

Research conclusions

Although patients with diabetes are more prone to developing all types of infections, their overall vaccination rate is still suboptimal. Adults with diabetes in Saudi Arabia have higher rate of COVID-19 and annual influenza vaccines compared to other vaccines recommended by CDC/ACIP. Among patients with diabetes, factors significantly influence the decision of vaccination include recommendations provided by family physicians or specialists, the site of care, income level, DM-related hospitalization history, residency site, HbA1c level, and health sector where care is being provided.

Research perspectives

This signifies the need for large-scale research to identify additional factors that might facilitate adherence to CDC/ACIP vaccine recommendations in patients with DM.

FOOTNOTES

Author contributions: Alqifari SF contributed to the conceptualization and project administration; Alqifari SF, Mutlaq MR, and Aldhaeefi M were involved in the methodology of this study; Alqifari SF, Amirthalingam P, and Alqahtani T analysed data; Alqifari SF, Esmail AK, Alarifi DM, Alsuliman GY, Alhati MM, Mutlaq MR, and Alshuaibi SA participated in the data interpretation; Alqifari SF, Esmail AK, Alarifi DM, Alsuliman GY, Alhati MM, Mutlaq MR, and Alshuaibi SA contributed to the writing - original draft preparation; Alqifari SF, Esmail AK, Mutlaq MR, Aldhaeefi M, Abdallah A, Wasel AS, Hamad HR, Alamin S, Atia TH, Alqahtani T took part in the writing - review and editing; Alqifari SF and Amirthalingam P were involved in the visualization; Alqifari SF, Mutlaq MR, Aldhaeefi M, and Alqahtani T participated in the supervision; and all authors have read and agreed to the published version of the manuscript.

Institutional review board statement: The study was conducted according to the guidelines of the Declaration of Helsinki. The study protocol (607-43-6007) was granted IRB approval by the Regional Research Ethics Committee - Ministry of Health, Saudi Arabia.

Informed consent statement: Informed consents have been obtained on first page of the online questionnaire. A clear information of the purpose of the study, participants rights during completing the questionnaire and withdrawal at any stage were provided. Only those who agreed to participate were able to complete the online survey.

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

Data sharing statement: The data that support the findings of this study are available on request from the corresponding author.

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ORIGINAL ARTICLE

Observational Study

Evaluation of hybrid closed-loop insulin delivery system in type 1 diabetes in real-world clinical practice: One-year observational study

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Abstract

BACKGROUND

In 2016, the Food and Drug Administration approved the first hybrid closed-loop (HCL) insulin delivery system for adults with type 1 diabetes (T1D). There is limited information on the impact of using HCL systems on patient-reported outcomes (PROs) in patients with T1D in real-world clinical practice. In this independent study, we evaluated glycemic parameters and PROs over one year of continuous use of Medtronic's 670G HCL in real-world clinical practice.

AIM

To assess the effects of hybrid closed loop system on glycemic control and quality of life in adults with T1D.

METHODS

We evaluated 71 patients with T1D (mean age: 45.5 ± 12.1 years; 59% females; body weight: 83.8 ± 18.7 kg, body mass index: 28.7 ± 5.6 kg/m^2, A1C: 7.6% ± 0.8%) who were treated with HCL at Joslin Clinic from 2017 to 2019. We measured A1C and percent of glucose time-in-range (%TIR) at baseline and 12 months. We measured percent time in auto mode (%TiAM) for the last two weeks preceding the final visit and assessed PROs through several validated quality-of-life surveys related to general health and diabetes management.

RESULTS

At 12 mo, A1C decreased by 0.3% ± 0.1% (P = 0.001) and %TIR increased by 8.1%

± 2.5% (P = 0.002). The average %TiAM was only 64.3% ± 32.8% and was not associated with A1C, %TIR or PROs. PROs, provided at baseline and at the end of the study, showed that the physical functioning submodule of 36Item Short-Form Health Survey increased significantly by 22.9% (P < 0.001). Hypoglycemia fear survey/worry scale decreased significantly by 24.9% (P < 0.000); Problem Areas In Diabetes reduced significantly by -17.2% (P = 0.002). The emotional burden submodules of dietary diversity score reduced significantly by -44.7% (P = 0.001). Furthermore, analysis of Clarke questionnaire showed no increase in awareness of hypoglycemic episodes. WHO-5 showed no improvements in subject's wellbeing among participants after starting the 670G HCL system. Finally, analysis of Pittsburgh Sleep Quality Index showed no difference in sleep quality, sleep latency, or duration of sleep from baseline to 12 mo.

CONCLUSION

The use of HCL in real-world clinical practice for one year was associated with significant improvements in A1C, %TIR, physical functioning, hypoglycemia fear, emotional distress, and emotional burden related to diabetes management. However, these changes were not associated with time in auto mode.

Key Words: Artificial pancreas; Continuous blood glucose monitor; Type 1 diabetes; Hybrid closed-loop insulin delivery; Quality of life

Core Tip: There has been a growing emphasis on patient-centered healthcare and there are limited data on the impact of hybrid-closed-loop systems on quality-of-life measures. In this study, we aimed to evaluate the glycemic control and quality of life measures in patients with type 1 diabetes in a real-world clinical practice who used hybrid closed loop systems showed improvements in A1C, percent time in optimal glucose range, emotional burden and distress due to diabetes, physical functioning, and fear of hypoglycemia.

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INTRODUCTION

Type 1 diabetes (T1D) affects around 1.6 million patients in the United States[1]. Despite many innovations in T1D management, reaching and maintaining optimal glycemic control remains difficult. The perpetual challenge of self-management and self-monitoring imposed by T1D are significant and put an enormous burden on patients. Moreover, discrepancies between food intake and insulin doses can result in severe and life-threatening acute complications (*e.g.*, severe hyperglycemia or severe hypoglycemia), along with the devastating long-term complications of uncontrolled diabetes, such as obesity, diabetic retinopathy, chronic kidney disease, non-alcoholic fatty liver disease, and cardiovascular disease[2-4]. These challenges call for more practical solutions, including the utilization of diabetes management technology[5]. Insulin pumps, used independently, or in combination with continuous glucose monitors (CGM), have been associated with better glycemic control and lower A1C[6-8]. The introduction of hybrid closed-loop (HCL) systems was a revolutionary step toward better glycemic control. These devices lessen the burden of diabetes self-management by adjusting insulin delivery based on real-time interstitial glucose values. Understanding patients' expectations from these devices is critical to ensure enhanced patient compliance and satisfaction[9,10].

Medtronic's MiniMed 670G (670G) was the first Food and Drug Administration-approved HCL insulin delivery system for patients with T1D[11]. It was followed by 3 other systems, MiniMed's 780G, Tandem's t:slim Control IQ and Insulet's Omnipod 5. In Auto Mode, the integrated CGM captures interstitial glucose values every five minutes, and *via* a built-in algorithm, it automatically adjusts basal insulin delivery, aiming at keeping glucose value around 120 mg/dL. During exercise, the algorithm adjusts glucose target to around 150 mg/dL. Meanwhile, pre-set basal insulin can be delivered throughout the day in manual mode[11,12].

Patients with T1D, who use HCL insulin delivery systems, have better glucose control and decreased risk of hypoglycemia, compared to those using independent sensor-augmented insulin pumps and a CGM[13]. The safety of HCL systems was demonstrated during in-home use by adolescents and adults. The results showed significant A1C reduction, higher percent of glucose time-in-range (%TIR), and lower percentage time in hyperglycemia or hypoglycemia compared to baseline[14,15].

However, data on the impact of HCL systems on quality-of-life measures are limited. Knowledge and understanding of this information are of particular importance due to growing emphasis on patient-centered healthcare. In this

independent prospective observational study, we evaluated clinical and patientreported outcomes (PROs) among patients with T1D who used 670G HCL system in real-world clinical practice over one year.

MATERIALS AND METHODS

Patients and methods

This study was approved by the Committee on Human Studies at the Joslin Diabetes Center. Each participant signed the study informed consent before enrollment in the 12-month observational study period.

We recruited 114 adult patients with T1D who started 670G HCL system at the Joslin Diabetes Adult Clinic between December 2017 and December 2019. Data were collected at baseline and after 12 months. We assessed PROs by administering the following surveys: 36Item Short-Form Health Survey (SF36)[16], Pittsburgh Sleep Quality Index (PSQI)[17], hypoglycemia fear survey/worry scale (HFS_W)[18], Problem Areas In Diabetes (PAID)[19], Well-Being Index (WHO-5)[20], Clarke hypoglycemia awareness survey and Diabetes Distress Scale with its sub sections: Emotional Burden, Physician-related Distress, Regimen-related Distress and Interpersonal Distress[21].

We measured A1C and %TIR at baseline and after 12 months of continuous use. We also evaluated percent time in auto mode (%TiAM) during the two weeks preceding the final study visit.

Out of the 114 participants in the study, 71 patients completed the 12-month follow-up and were included in this final analysis.

Statistical analysis

Demographic and baseline characteristics were expressed as mean ± SD or as mean (95% confidence interval). Categorical variables were expressed as percentages. Chi-square test and paired t-test were used to compare endpoints between baseline and at 12 mo. A P value of < 0.05 was considered statistically significant. All analyses were performed using STATA Special Edition 15.0 for Windows® (StataCorp®, College Station, Texas, United States, 2017).

RESULTS

In this study, we evaluated 71 patients with T1D (mean age: 45.5 ± 12.1 years' 59% females' body weight: 83.8 ± 18.7 kg, body mass index: 28.7 ± 5.6 kg/m^2, A1C: 7.6 ± 0.8%; Table 1).

At 12 mo, A1C decreased by 0.3% ± 0.1% (P = 0.001) and %TIR increased by 8.1% ± 2.5% (P = 0.002; Table 2). The average %TiAM was only 64.3% ± 32.8% and was not associated with A1C, %TIR or PROs at both, the beginning and end of the study.

PROs, provided at baseline and at the end of the study, showed that the physical functioning submodule of SF-36 increased significantly by 22.9% (P < 0.001), with no significant differences observed in other submodules of SF-36. HFS_W decreased significantly by 24.9% (P < 0.001); PAID reduced significantly by -17.2% (P = 0.002); Overall, total dietary diversity score (DDS) was not reduced significantly, but emotional burden submodules of DDS reduced significantly by -44.7% (P < 0.001). Furthermore, analysis of Clarke questionnaire showed no increase in awareness of hypoglycemic episodes. WHO-5 showed no improvements in subject's wellbeing among participants after starting the 670G HCL system. Finally, analysis of PSQI showed no difference in sleep quality, sleep latency, or duration of sleep from baseline to 12 months.

DISCUSSION

In this study, we prospectively followed 71 patients with T1D who started HCL insulin delivery system (Medtronic's MiniMed 670G) for 12 mo in real-world clinical practice. The study showed that glycemic parameters improved significantly where A1C decreased by 0.3% ± 0.1% (P < 0.001), and glucose %TIR increased by 8.1% ± 2.5% (P = 0.002). The improvement in glycemic parameters were associated with improvement in some PROs, including PAID, HFS_W, emotional burden and interpersonal distress submodules of DDS-significant increase in the SF-36 physical functioning score. However, neither of these changes were associated with the %TiAM, which was only 64.3% ± 32.8% of the time wearing the HCL system. The study also showed no improvement in subjects' wellbeing and no difference in sleep quality, sleep latency, or duration of sleep from baseline to 12 months.

The improvement in glycemic parameters in real-world clinical practice are aligned with previous observation on the 670G HCL system in clinical research studies[15,22,23]. In a pivotal MiniMed 670G clinical study, the reduction in A1C ranged from 0.5% to 0.7%[24]. Here, we showed a smaller decrease in A1C of 0.3%. A potential explanation for this difference could be related to the discrepancy in the %TiAM, which was 87% in the pivotal study, in comparison to 64.3% ± 32.8% in this study. Although this could be a logical explanation for the discrepancy in glycemic improvement, our study showed no relationship between glycemic parameters and %TiAM. We could postulate that sensor fatigue and suboptimal follow up in real-world clinical practice played some role. Patients enrolled in clinical trials are generally under close-monitoring and are provided with better support.

Table 1 Baseline characteristics of patients

Variable	Whole cohort (*n* = 71)
Female, *n* (%)	42 (59)
Age (yr)	45.5 ± 12.1
Weight (kg)	83.8 ± 18.7
BMI (kg/m²)	28.7 ± 5.6
Diabetes duration (yr)	30.0 ± 12.7
HbA1c (%)	7.6 ± 0.8

Data are mean ± SD or *n* (%).

Table 2 Changes to glycemic and quality of life parameters after 12 months of using Hybrid-Closed-loop system

	% change from baseline	*P* value[1]
Glycemic parameters (%)		
HbA1c	-0.3	0.001
Time in range	+8.1	0.002
Participant reported outcomes		
SF 36		
Physical functioning	+22.9	< 0.001
Role functioning/physical	-6.3	0.2
Role functioning/emotional	-1.2	0.8
Energy/Fatigue	+1.4	0.6
Emotional well-being	-0.2	0.9
Social functioning	-1.0	0.6
Pain	-1.4	0.6
General health	-0.9	0.7
DDS	-5.6	0.1
Emotional Burden	-44.7	< 0.001
Physician-related Distress	-5.9	0.7
Regimen-related Distress	-5.0	0.2
Interpersonal Distress	-10.5	0.1
PSQI	-1.6	0.8
HFS-W	-24.9	< 0.001
Clarke Hypoglycemia Awareness Survey	+9.5	0.2
WHO-5 Well-Being Index	-5.6	0.2
PAID	-17.2	0.002

[1]Paired *t*-test.
Data are mean percentage change from baseline.
SF36: Short Form-36; DDS: Diabetes Distress Scale; PSQI: Sleep Quality Assessment; HFS-W: Hypoglycemic Fear Survey, Worry subscale; PAID: Problem Areas in Diabetes.

Before this study, there were limited data on the impact of HCL on PROs. Therefore, this study may be of particular importance, since it evaluated significant number of quality-of-life parameters. It is known that the psychological and behavioral aspects of patients who have T1D for long duration significantly influence user adaptation to new diabetes technology. Interestingly, our study showed contradicting results with previous studies[25,26]. McAuley et.al. assessed HCL against usual care in adults with T1D[25]. Their study showed that people on HCL had better diabetes-specific well-being and quality of life without a change in either diabetes distress or treatment satisfaction, which might be explained by the burden of adopting new technologies[25]. Wheeler *et al*[26] conducted a randomized crossover trial, in which they assessed sleep quality and technology satisfaction with using HCL compared to sensor Augmented Pump therapy with Predictive Low Glucose Management in people with T1D. Their study showed a statistically significant improvement in quality of sleep and treatment satisfaction. However, the general psychological health and the worry associated with hypoglycemia persisted. On the contrary, our study showed no significant differences in sleep quality, sleep latency and duration in PSQI. We were expecting an improvement in sleep quality due to reduced episodes and/or alarms for hypoglycemia and decreased requirements for checking blood glucose when patients are symptomatic. In fact, the increased frequency of CGM alarms to calibrate in-order to put the HCL system back into Auto Mode, could be the main reason for lack of improvement in sleep quality.

This study had several limitations. The study lacks social diversity, as it was conducted in a single, tertiary-care center, where majority of participants are well-educated. This might have had an impact on the patients' adoption of new technologies, which might not reflect the same conditions in the general population. Several studies have shown the benefit of technological advancements; such as pumps, devices, and virtual interventions on diabetes management, but also report patients' adoption of technologies as a potential limitation[27,28]. PROs were paper-based, which was convenient for patients to complete and minimized technological barriers but was subject to human errors when transferred electronically from paper forms. Nevertheless, a recent meta-analysis evaluating bias in mode of administering PROs found no bias between paper-based and electronic-based methods[29]. Another limitation is that training patients on PROs was briefly addressed during study initiation. This might have contributed to inconsistency and confusion surrounding some of the provided questionnaires. Also, the lack of a run-in period for device training and incomplete information about participants' history of CGM use further limited this study. Considering that HCL is a newer technology, it comes with the usual burden of participants' adoption and adaptation, which may vary significantly between patients. Furthermore, future research should focus on collecting PROs from a larger sample size, while implementing ample training opportunities to ease the burden of adapting newer technologies. Similar studies are required to evaluate PROs for newer HCL systems; Omnipod 5 and t:slim Control IQ since these HCL systems include a CGM that does not require calibration and could achieve greater %TiAM and possibly improve sleep quality. Another limitation is the lack of data on the type and delivery method of insulin prior to starting the study, as such data could interfere with the outcomes of the study or the effect of the HCL. This independent study from a specialized diabetes center may help industry to improve diabetes technology used for insulin delivery.

CONCLUSION

the use of HCL insulin delivery system in real-world clinical practice results in significant improvements in A1C. This study showed considerable improvements in physical functioning, emotional functioning, and emotional adjustment to various aspects of diabetes management compared to baseline. It also showed that fear of diabetes management over time and the feeling of inappropriate support from family and friends were significantly less. Meanwhile, it showed that the use of HCL is also associated with reduction in fear of hypoglycemic episodes but with no increase in awareness of hypoglycemic episodes. Despite improvement in many PROs, participants' subjective sense of well-being did not show any improvement after starting HCL.

ARTICLE HIGHLIGHTS

Research background

Technology has been playing an increasing role in the management of diabetes. The introduction of hybrid closed-loop (HCL) systems and continuous glucose monitors (CGM) was a revolutionary step toward better glycemic control. However, there is limited data on the impact of HCL on patient-reported outcomes (PROs).

Research motivation

Data on the impact of HCL systems on quality-of-life measures are limited. Knowledge and understanding of this information are of particular importance due to growing emphasis on patient-centered healthcare. This study from a specialized diabetes center may help future research to improve diabetes technology used for insulin delivery.

Research objectives

In this independent prospective observational study, we evaluated clinical and PROs among patients with T1D who used HCL system in real-world clinical practice over one year.

Research methods
Participants with T1D who were treated with HCL at Joslin Clinic from 2017 to 2019 were evaluated. We measured A1C and percent of glucose time-in-range (%TIR) at baseline and 12 months. We measured percent time in auto mode or the last two weeks preceding the final visit and assessed PROs through several validated quality-of-life surveys related to general health and diabetes management.

Research results
At 12 months, A1C decreased by 0.3% ± 0.1% and %TIR increased by 8.1% ± 2.5%. The physical functioning submodule of 36Item Short-Form Health Survey increased significantly by 22.9%. Hypoglycemia fear survey/worry scale decreased significantly by 24.9%; Problem Areas In Diabetes reduced significantly by -17.2%. The emotional burden submodules of dietary diversity score reduced significantly by -44.7%.

Research conclusions
The implementation of HCL in care of T1D in real-world clinical practice for one year is associated with significant improvements in A1C, %TIR, physical functioning, hypoglycemia fear, emotional distress, and emotional burden related to diabetes management.

Research perspectives
Future research should focus on better understanding the effects of HCL system on the patients with diabetes. Larger cohorts are needed for the validation of these results and clinical care should take these outcomes into considerations when deciding on appropriate management for patients.

FOOTNOTES

Author contributions: Eldib A, Dhaver S, Kibaa K and Hamdy O have full access to all study data and take responsibility for data integrity and accuracy of data analysis; Eldib A designed the study, collected data, conducted the statistical analysis, and prepared the manuscript; Dhaver S and Kibaa K collected data, conducted the statistical analysis, and prepared the manuscript; Atakov-Castillo A collected data and reviewed the manuscript; Al-Badri M, Salah T, Khater A, McCarragher R, Elenani O prepared, reviewed, and edited the manuscript; Toschi E and Hamdy O designed and supervised the study, reviewed, and edited the manuscript; all authors approved the final version of the manuscript.

Institutional review board statement: The study was reviewed and approved by the Committee on Human Studies at the Joslin Diabetes Center (Approval No. 2017-14).

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

Conflict-of-interest statement: Eldib A, Dhaver S, Kibaa K, Atakov-Castillo A, Salah T, Al-Badri M, Khater A, McCarragher R, Elenani O, Toschi E: Nothing to disclose. Hamdy O: Consultant to Abbott Nutrition, Sanofi Aventis; his employer Joslin Diabetes Center receives research grants from Novo-Nordisk, Eli-Lilly, Gilead Sciences, and National Dairy Council; on SAB of Twin Health; and is a shareholder of Healthimation Inc.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at tareq.salah@joslin.harvard.edu. No additional data are available.

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ORIGINAL ARTICLE

Observational Study

Comparative efficacy of sodium glucose cotransporter-2 inhibitors in the management of type 2 diabetes mellitus: A real-world experience

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Abstract

BACKGROUND

Sodium glucose cotransporter-2 inhibitors (SGLT-2i) are a class of drugs with modest antidiabetic efficacy, weight loss effect, and cardiovascular benefits as proven by multiple randomised controlled trials (RCTs). However, real-world data on the comparative efficacy and safety of individual SGLT-2i medications is sparse.

AIM

To study the comparative efficacy and safety of SGLT-2i using real-world clinical data.

METHODS

We evaluated the comparative efficacy data of 3 SGLT-2i drugs (dapagliflozin, canagliflozin, and empagliflozin) used for treating patients with type 2 diabetes mellitus. Data on the reduction of glycated hemoglobin (HbA1c), body weight,

blood pressure (BP), urine albumin creatinine ratio (ACR), and adverse effects were recorded retrospectively.

RESULTS

Data from 467 patients with a median age of 64 (14.8) years, 294 (62.96%) males and 375 (80.5%) Caucasians were analysed. Median diabetes duration was 16.0 (9.0) years, and the duration of SGLT-2i use was 3.6 (2.1) years. SGLT-2i molecules used were dapagliflozin 10 mg (n = 227; 48.6%), canagliflozin 300 mg (n = 160; 34.3%), and empagliflozin 25 mg (n = 80; 17.1). Baseline median (interquartile range) HbA1c in mmol/mol were: dapagliflozin - 78.0 (25.3), canagliflozin - 80.0 (25.5), and empagliflozin - 75.0 (23.5) respectively. The respective median HbA1c reduction at 12 months and the latest review (just prior to the study) were: 66.5 (22.8) & 69.0 (24.0), 67.0 (16.3) & 66.0 (28.0), and 67.0 (22.5) & 66.5 (25.8) respectively (P < 0.001 for all comparisons from baseline). Significant improvements in body weight (in kilograms) from baseline to study end were noticed with dapagliflozin - 101 (29.5) to 92.2 (25.6), and canagliflozin 100 (28.3) to 95.3 (27.5) only. Significant reductions in median systolic and diastolic BP, from 144 (21) mmHg to 139 (23) mmHg; (P = 0.015), and from 82 (16) mmHg to 78 (19) mmHg; (P < 0.001) respectively were also observed. A significant reduction of microalbuminuria was observed with canagliflozin only [ACR 14.6 (42.6) at baseline to 8.9 (23.7) at the study end; P = 0.043]. Adverse effects of SGLT-2i were as follows: genital thrush and urinary infection - 20 (8.8%) & 17 (7.5%) with dapagliflozin; 9 (5.6%) & 5 (3.13%) with canagliflozin; and 4 (5%) & 4 (5%) with empagliflozin. Diabetic ketoacidosis was observed in 4 (1.8%) with dapagliflozin and 1 (0.63%) with canagliflozin.

CONCLUSION

Treatment of patients with SGLT-2i is associated with statistically significant reductions in HbA1c, body weight, and better than those reported in RCTs, with low side effect profiles. A review of large-scale real-world data is needed to inform better clinical practice decision making.

Key Words: Sodium glucose cotransporter-2 inhibitors; Empagliflozin; Canagliflozin; Dapagliflozin; Type 2 diabetes mellitus; Cardiovascular disease; Albumin creatinine ratio; Diabesity

Core Tip: Treatment of type 2 diabetes mellitus with sodium glucose cotransporter-2 inhibitors (SGLT-2i) is associated with significant glycated hemoglobin (HbA1c) reduction, body weight loss, and cardiovascular benefits as proven by multiple randomised controlled trials (RCTs). Our real-world data analysis of the efficacy and safety of individual SGLT-2i revealed better reduction of HbA1c with dapagliflozin, canagliflozin, and empagliflozin, while better body weight reduction was seen only with dapagliflozin and canagliflozin when compared to RCTs. Blood pressure reduction, and side effect profiles were comparable to previous studies. A significant improvement of albuminuria was obvious only with canagliflozin, presumably because of the low number of participants in this study. Analyses of various large-scale real-world data are expected to inform better clinical practice decision making in future.

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INTRODUCTION

Sodium glucose cotransporter-2 inhibitors (SGLT-2i) belong to a class of new antidiabetic drugs with mainly extra-pancreatic mechanism of actions to control hyperglycemia predominantly by enhancing renal glycosuria. Even in nondiabetic individuals, these agents can cause a daily loss of about 80 g of glucose through the kidneys, which is much higher in individuals with hyperglycemia, and therefore, SGLT-2i possess moderate antidiabetic efficacy[1]. Glycosuria also results in a net body energy deficit (the reason why SGLT-2i causes weight loss), and osmotic diuresis as consequence - the additional benefits of these medications. The weight loss benefit makes this class of drugs an attractive treatment option for patients with type 2 diabetes mellitus (T2DM) and diabesity - diabetes because of obesity[2,3]. Moderate improvement in glycemic control with a reduction in glycated hemoglobin (HbA1c) is seen with the use of these drugs[1-11]. Multiple randomised controlled trials (RCTs) and meta-analyses have proven that SGLT-2i use is associated with cardiovascular benefits not only for primary prevention but also for the management of established cardiovascular disease[4-11]. Multiple studies also revealed the remarkable nephroprotective effects of this class of medications[10-14].

Although there are multiple clinical trials and prospective cohort studies examining the cardiovascular, renal, and diabesity outcomes of individual SGLT-2i molecules, making them drugs of choice in day-to-day management of patients

with T2DM and multiple comorbidities related to diabesity, the comparative data on the efficacy of each molecule are rather sparse in the global scientific literature. As clinical trial and prospective cohort study settings are appropriately supervised and often rigorously controlled by the study teams, the data captured by these methods may not always reflect the actual real-world clinic picture when we consider the study outcomes. Therefore, we should have adequate real-world data from our own day-to-day clinical practice to truly appraise the actual long-term benefits of SGLT-2i and to help therapeutic decision making for our T2DM patients with multi-comorbidities. The current study was designed to suit this purpose in a large academic teaching hospital in the United Kingdom.

MATERIALS AND METHODS

Study design & settings

This is a retrospective clinical study by review of the data of all patients treated with any one of the SGLT-2i drug, empagliflozin, canagliflozin, or dapagliflozin between 1st July 2016 and 30th June 2022 at Lancashire Teaching Hospitals NHS Trust. The diabetes service of this hospital provides comprehensive diabetic care for patients in the Central Lancashire and South Cumbria regions of the United Kingdom, catering a population of about 0.4 million people. The study was approved by the institutional research committee (No: KB/PB/SE-387/2022).

SGLT-2i molecules used and the dosages

The standard practice of using the SGLT-2i molecules in the hospital was to start with lower doses (daily dose of dapagliflozin at 5 mg, empagliflozin at 10 mg and canagliflozin at 100 mg) and subsequently to increase the dose to full strength of the drug (dapagliflozin 10 mg, empagliflozin 25 mg, and canagliflozin 300 mg, respectively) after a month with monitoring of renal functions just prior to the dose increment.

Participants

All adult patients (age ≥ 18 years) with T2DM treated by one of the above three SGLT-2i molecule for management of their diabetes were considered for inclusion in the study.

Data capture

Electronic records were searched for all patients with a diagnosis of DM treated with antidiabetic medications of the SGLT-2i class during the study period. The total number of cases in this category was further reviewed for inclusion in the study.

Inclusion criteria

Patients with a diagnosis of T2DM managed with one of the SGLT-2i. Participants with predefined primary outcome measures (HbA1c alteration from baseline values at follow-up) and/or secondary outcomes such as alterations in body weight ± body mass index (BMI), blood pressure (BP), renal functions, and urine albumin creatinine ratio (ACR) - all these outcomes with meaningful data in at least one of the follow-up period (6 months, 12 months and/or at the last follow up just prior to the study), and/or the development of adverse events [such as genital thrush, urinary tract infections (UTI) or diabetic ketoacidosis (DKA)].

Exclusion criteria

Patients with T1DM. Incomplete study data to obtain meaningful outcome measures as specified above. Follow up duration less than 6 months. SGLT-2i commenced primarily for patients for other indications (such as heart failure or CKD) without T2DM or diet controlled T2DM. Discontinuation of SGLT-2i within 6 months of initiation for any reason including major adverse events such as DKA, recurrent genital thrush, allergy reactions or UTI.

Data management

Two junior doctors, under the supervision of a senior academic, recorded the relevant study information using a Microsoft Exel data sheet by entering the demographic, clinical, biochemical, and follow-up data of all study subjects from their electronic case records retrieved with the help of a senior data scientist of the hospital.

Statistical analysis

Initially, each quantitative trait was tested for normality of distribution using the Shapiro-Wilk normality test. Continuous variables were expressed as median and interquartile range (IQR), and categorical variables as numbers and percentages. The nonparametric Wilcoxon signed-rank test was used for within-group quantitative differences, whereas the two-tailed Fisher's exact test was used to compare proportions. Spearman's rank correlation analysis was used to explore the correlation between the safety and efficacy of SGLT-2 inhibitors with clinical and biochemical parameters. Kruskal Wallis nonparametric test was used to compare the relationship between continuous variables in different SGLT-2i drugs. The χ^2 test is used for finding the association between two categorical variables. A P-value of < 0.05 (two-tailed) was considered statistically significant. Data were analyzed with Jamovi Software, Version 2.3.21.0 (based on the R program) and BlueSky Statistics Version 10.0, R package version 8.0.

RESULTS

Clinical and biochemical baseline characteristics of SGLT-2i-treated patients

Among a total of 562 patients treated with SGLT-2i during the study period, 467 T2DM patients had meaningful data for analysis of primary and or secondary outcomes. Table 1 shows the main clinical and biochemical characteristics of these subjects. Out of these patients, 160 (34.3%) were on canagliflozin, 227 (48.6%) on dapagliflozin, and 80 (17.1%) on empagliflozin. All these patients were using the full standard recommended doses of individual SGLT-2i molecules.

Efficacy of SGLT-2i on glycemic control (primary outcome)

The median duration of SGLT-2i therapy was 3.6 (2.1) years. An overall median HbA1c reduction from 78 (26) mmol/mol to 68 (26) mmol/mol ($P < 0.001$) was observed with the drug treatment. In other words, we observed a median HbA1c reduction of -10 (IQR 25) mmol/mol, with a percentage decrease of 12.82% from the baseline value, indicating moderate antidiabetic efficacy of this class of oral agents. During this treatment period, a significant reduction in HbA1c levels was observed, with a more pronounced decrease in HbA1c during the first six months of SGLT-2i use.

Secondary outcomes of SGLT-2i treatment

Considering the secondary outcomes, a median body weight reduction from 99 (29) kg to 95.5 (30.3) kg; ($P < 0.001$) in the first 6 months, with a further reduction to 92.4 kg (median with IQR 27.3) at the latest measurement, and this decrease paralleled the median reduction in BMI: from 34 (7.58) to 33 (7.47) kg/m^2 ($P < 0.001$). Significant reductions in median systolic BP, from 144 (21) mmHg to 139 (23) mmHg ($P = 0.015$), and median diastolic BP from 82 (16) mmHg to 78 (19) mmHg ($P < 0.001$) were also observed. Although a significant reduction in median estimated glomerular filtration rate (eGFR) from 88 (17) mL/min to 86.5 (18) mL/min ($P < 0.001$) at the 6 months' follow up after the SGLT-2i commencement, an improvement to 87 (18) mL/min at 12 months, and maintenance at that level median eGFR: 87 (21) mL/min at latest follow up was noticed. No statistically significant differences were observed in other biochemical parameters like serum creatine and gamma glutamyl transpeptidase were observed at any follow up periods.

Treatment outcomes with individual SGLT-2i molecules

The primary outcome (HbA1c reduction) with each SGLT-2i drug is shown in Figure 1 and the composite treatment outcomes in Tables 2-4. The median weight changes with the individual SGLT-2i drug molecule are shown in Figure 2 and the gender-specific latest weight is shown in the Figure 3.

Subgroup analysis

Only 98 participants had microalbuminuria at baseline and meaningful follow up data of urine ACR for analysis. Of these subjects, only those on canagliflozin showed a statistically significant reduction of albuminuria at the latest follow up, though dapagliflozin showed a significant reduction in ACR at 6 and 12 months follow up periods (Figure 4).

Adverse events of treatment

A total of 64 (13.7%) participants developed adverse events with the use of SGLT-2i therapy [genital thrush in 46 (9.9%), UTI in 11 (2.36%), and DKA in 5 (1.07%) cases]. Adverse effects of individual SGLT-2i molecules were as follows: Genital thrush and urinary infection - 20 (8.8%) & 17 (7.5%) participants with dapagliflozin; 9 (5.6%) & 5 (3.13%) participants with canagliflozin; and 4 (5%) & 4 (5%) participants with empagliflozin. DKA was observed in 4 (1.8%) participants with dapagliflozin and 1 (0.63%) participant with canagliflozin. No participant on empagliflozin developed DKA.

Risk factor for DKA

Explanatory data analysis using box plot showed that a high baseline and follow-up HbA1c levels were correlated with higher risk for DKA (Figure 5). While a baseline median HbA1C of ≥ 109 (18) mmol/mol was associated with higher risk of DKA, an HbA1C of < 78 (25) mmol/mol is appeared to confer protection from DKA. Similarly, median HbA1c at 12 months ≥ 81 (4) mmol/mol was associated with DKA, HbA1c level at 12 months < 67 (20) mmol/mol was associated with protection from DKA.

DISCUSSION

In this retrospective cohort study involving 467 participants with a median duration of T2DM of 16 (9) years treated for a median duration of 3.6 (2.1) years with various SGLT2i medications, we observed statistically significant improvements in glycemic, metabolic, and other cardiovascular outcomes known to occur with the use of this class of drugs in previous clinical studies. The median HbA1c reduction observed from baseline was 10-14 mmol/mol with these drug use at various follow up periods. Significant and persistent improvements in body weight from baseline to the study end were noticed with dapagliflozin (8.8 kg) and canagliflozin (4.7 kg). Though empagliflozin use was associated with significant weight loss at six (6 kg) and 12 months (9.2 kg) there was a regain of weight (see Table 4) at latest follow up. Significant reduction of microalbuminuria was observed with canagliflozin only [ACR 14.6 (42.6) at baseline to 8.9 (23.7) at study end]. The observed adverse effects with the use of SGLT-2i were not unacceptably high in our study. High baseline and follow up HbA1c levels predicted the risk of ketoacidosis.

Table 1 Baseline clinical and biochemical characteristics of 467 type 2 diabetes mellitus patients treated with sodium glucose cotransporter 2 inhibitors.

Baseline features	*n* (%) or median (IQR)
Female gender	173 (37.04%)
Age (yr)	64 (14.8)
Ethnicity	
British white	338 (72.37%)
Diabetes duration (yr)	16 (9)
Diabetes duration ≥ 10 yr	335 (71.73%)
Years of treatment with SGLT2i	3.6 (2.1)
Body weight (kg)	99 (29)
BMI (kg/m^2)	34 (7.58)
BP (mmHg)	
Systolic	144 (21)
Diastolic	82 (16)
HbA1c (mmol/mol)	78 (25.5)
Serum creatinine (μmol/L)	74 (21)
eGFR (mL/min/m^2)	88.5 (17)
Urine ACR	2.1 (3.95)
GGT	38 (0)
Concomitant medications	
Basal-bolus (novorapid + long-acting insulin)	32 (18.7)
Mixed insulin (novomix-30 insulin)	53 (26.4)
Long-acting insulin (lemevir, toujeo, lantus, tresiba, absalgar)	83 (38.1)
Metformin	377 (83.9)
Sulfonylurea (glimepride/glyburide/glipizide)	199 (44.7)
DPP4 inhibitors (sitagliptin, saxagliptin, linagliptin, or alogliptin)	130 (29.1)
Pioglitazone	22 (5)
GLP1 inhibitor (dulaglutide, exanatide, semaglutide, liraglutide)	94 (20.9)

SGLT2i: Sodium glucose cotransporter-2 inhibitor; IQR: Interquartile range; BMI: Body mass index; BP: Blood pressure; HbA1c: Glycated hemoglobin; eGFR: Estimated glomerular filtration rate; ACR: Albumin creatinine ratio; GGT: Gamma glutamyl transpeptidase; DPP4: Dipeptidyl-peptidase 4; GLP1: Glucagon like peptide 1.

Dapagliflozin was the commonest SGLT-2i molecule used in our cohort. A recent meta-analysis using pooled data from 8 clinical trials involving 3747 patients showed that its use is associated with a mean HbA1c reduction of 0.59% (approximately 7 mmol/mol)[15] though the first clinical trial (as an add-on therapy to metformin) with the molecule showed a better HbA1c reduction of 0.84% (approximately 9.5 mmol/mol)[16]. We observed a median HbA1c reduction of 10, 11.5 and 9 mmol/mol respectively at 6, 12 and last (median 3.6 years) follow up periods indicating a better treatment response than that were seen in the clinical trials. The better efficacy in glycemic control we observed may be related to the poorer baseline glycemic control (high baseline HbA1c) in our patients, a usual feature in the real-world settings compared to the strictly controlled clinical trial settings where some degree of selection bias is expected with participants likely having better baseline glycemic parameters. Moreover, a significant chunk of our patients was already on multiple antidiabetic agents at the time of addition of dapagliflozin where some drug synergism is expected. The efficacy of dapagliflozin appears to be better even in the clinical trial settings when baseline glycemic control is poor (with an observed mean HbA1c reduction of 12 mmol/mol at 24 wk), and the drug is used as an add-on therapy as observed in a recent study[17].

We observed much better weight reduction with the use of dapagliflozin in our cohort (median -8.8 kg at last follow up) compared to a weight reduction observed in the meta-analysis (-1.88 kg) by Pinto *et al*[15]. More profound weight loss observed in our study may be related to the poor baseline glycemic control in our cohort and multicombination antidiabetic drug therapy. Patients with poorer diabetes control are likely to have more profound glycosuria and the

Table 2 Follow-up metabolic/clinical/lab parameters for dapagliflozin

Clinical/lab/parameter	Baseline, median (IQR; *n*)	6 months F/U, median (IQR; *n*)	12 months, median (IQR; *n*)	Latest, median (IQR; *n*)	*P* value (baseline *vs* latest)
HbA1c	78 (25.3; 227)	68 (25; 204)[a]	66.5 (22.8; 190)[b]	69 (24; 191)	< 0.001
Weight	101 (29.5; 198)	95.4 (28.2; 150)[a]	95.9 (29.2; 144)[a]	92.2 (25.6; 93)	< 0.001
BMI	33.7 (6.6; 81)	-	-	33.9 (6.38; 44)	0.002
Systolic BP	145 (21.3; 185)	-	142 (23; 159)	142 (23.3; 112)	0.080
Diastolic BP	84 (14; 185)	-	80 (14; 159)[b]	80 (19.3; 112)	< 0.001
Albuminuria (> 3 mmol/mg)	5.2 (17.4; 39)	3 (14.9; 24)[a]	2.1 (6.38; 18)[a]	4.4 (11.9; 30)	0.063

[a]*P* < 0.05.
[b]*P* < 0.01.
BMI: Body mass index; BP: Blood pressure; HbA1c: Glycated hemoglobin; IQR: Interquartile range.

Table 3 Follow-up metabolic/clinical/lab parameters for canagliflozin

Clinical/lab/parameter	Baseline, median (IQR; *n*)	6 months F/U, median (IQR; *n*)	12 months, median (IQR; *n*)	Latest, median (IQR; *n*)	*P* value (baseline *vs* latest)
HbA1c	80 (25.5; 160)	67.5 (20; 132)[b]	67 (16.3; 128)[b]	66 (28; 135)	< 0.001
Weight	100 (28.3; 125)	97.6 (27.5; 91)[a]	98.3 (30.4; 74)	95.3 (27.5; 58)	< 0.001
BMI	35.1 (8.1; 73)	-	-	32.5 (9.38; 50)	< 0.001
Systolic BP	145 (18; 121)	-	145 (21.3; 84)	138 (22.8; 64)	0.041
Diastolic BP	84 (18; 121)	-	80.5 (11.2; 84)[a]	79.5 (19; 64)	0.013
Albuminuria (> 3 mmol/mg)	14.1 (42.6; 40)	17.1 (30.4; 12)	7.3 (12.3; 21)[a]	8.9 (23.7; 28)	0.043

[a]*P* < 0.05.
[b]*P* < 0.01.
BMI: Body mass index; BP: Blood pressure; HbA1c: Glycated hemoglobin; IQR: Interquartile range.

Table 4 Follow-up metabolic/clinical/lab parameters for empagliflozin

Clinical/lab/parameter	Baseline, median (IQR; *n*)	6 months F/U, median (IQR; *n*)	12 months, median (IQR; *n*)	Latest, median (IQR; *n*)	*P* value (baseline *vs* latest)
HbA1c	75 (23.5; 80)	64.5 (25; 64)[b]	67 (22.5; 59)[a]	66.5 (25.8; 66)	0.002
Weight	92 (27.5; 57)	86 (22.8; 29)[a]	82.8 (20.9; 20)[b]	89.7 (27.5; 20)	0.207
BMI	33.7 (9.92; 24)	-	-	32.4 (5.57; 6)	0.10
Systolic BP	136 (24.3; 52)	-	137 (26; 23)	130 (25.3; 32)	0.096
Diastolic BP	74 (14.5; 52)	-	74 (16; 23)	75.5 (12.5; 32)	0.187
Albuminuria (> 3 mmol/mg)	6.4 (15; 19)	6.2 (11.8; 7)	3.2 (2.98; 6)	5.9 (2.35; 11)	0.79

[a]*P* < 0.05.
[b]*P* < 0.01.
BMI: Body mass index; BP: Blood pressure; HbA1c: Glycated hemoglobin; IQR: Interquartile range.

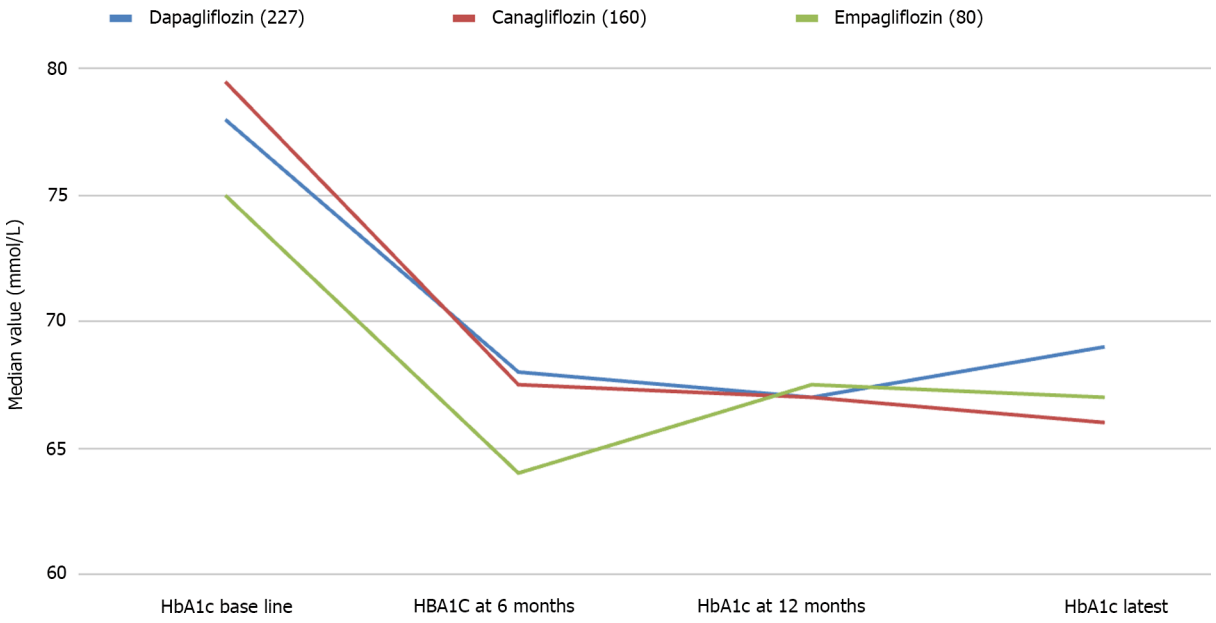

Figure 1 Changes of glycated hemoglobin from baseline at 6 months, 12 months, and latest with various sodium glucose cotransporter 2 inhibiting agents: Dapagliflozin, canagliflozin, and empagliflozin. HbA1c: Glycated hemoglobin.

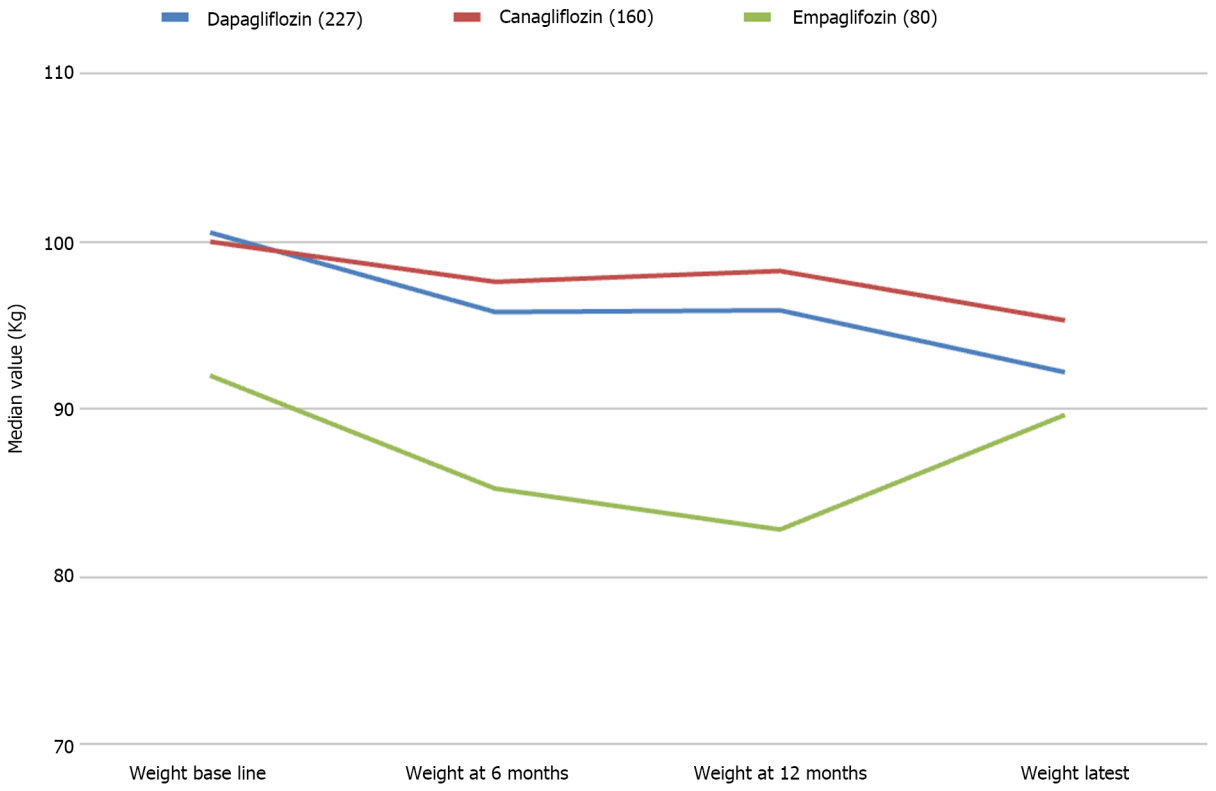

Figure 2 Weight changes, from baseline to latest follow up period with dapagliflozin, canagliflozin, and empagliflozin.

related negative energy balance from SGLT-2i as evidenced by previous studies with greater weight loss and better average HbA1c reduction as a consequence[18-20], supporting our observations. Although the systolic BP reduction following treatment did not reach statistical significance (P = 0.08) possibly because of the low number of participants in our cohort, a tendency towards significance in this study supports the beneficial effects of dapagliflozin as shown by other studies[21,22]. The significant reduction in diastolic BP observed in this cohort is also in agreement with the observations from other studies[23,24]. Although we observed significant reduction of albuminuria as observed by the DAPA-CKD trial[25], at 6 months and 12 months of follow up, the ACR reduction at the latest follow up did not reach statistical significance (P = 0.06) presumably because of the relatively low number of participants in the cohort. The adverse events such as genital thrush and UTI were relatively high from dapagliflozin use as shown by a recent meta-

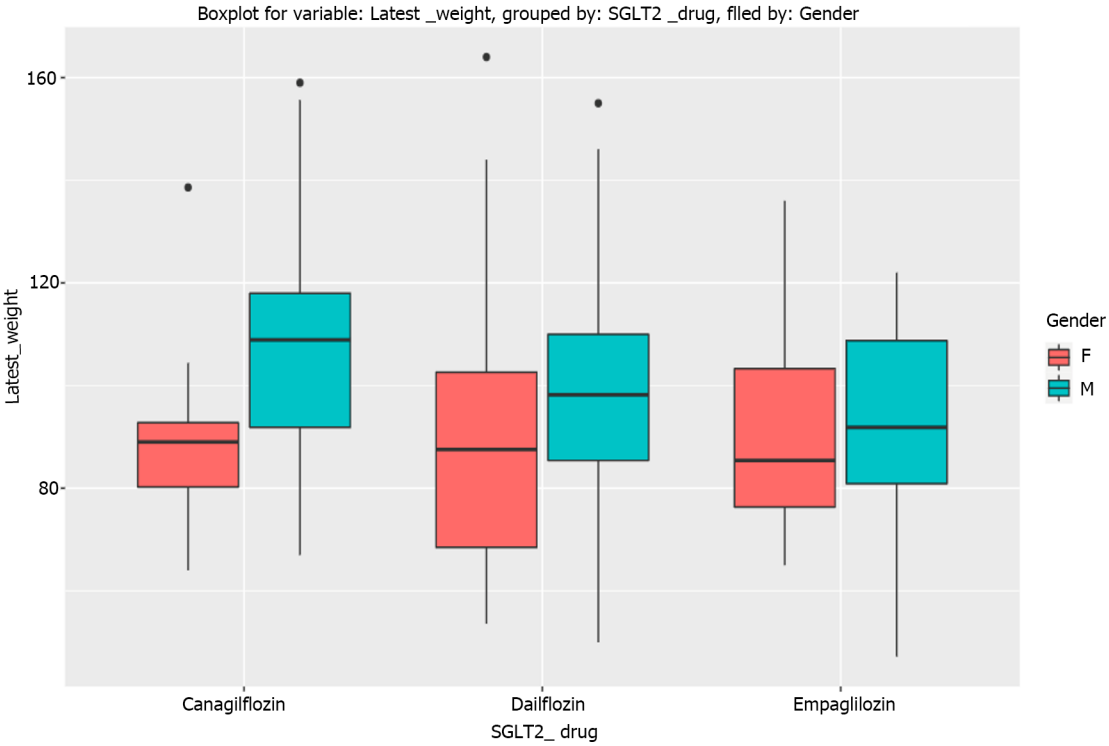

Figure 3 Boxplots showing latest weight, with dapagliflozin, canagliflozin, and empagliflozin in male and female patients. SGLT2: Sodium glucose cotransporter 2; F: Female; M: Male.

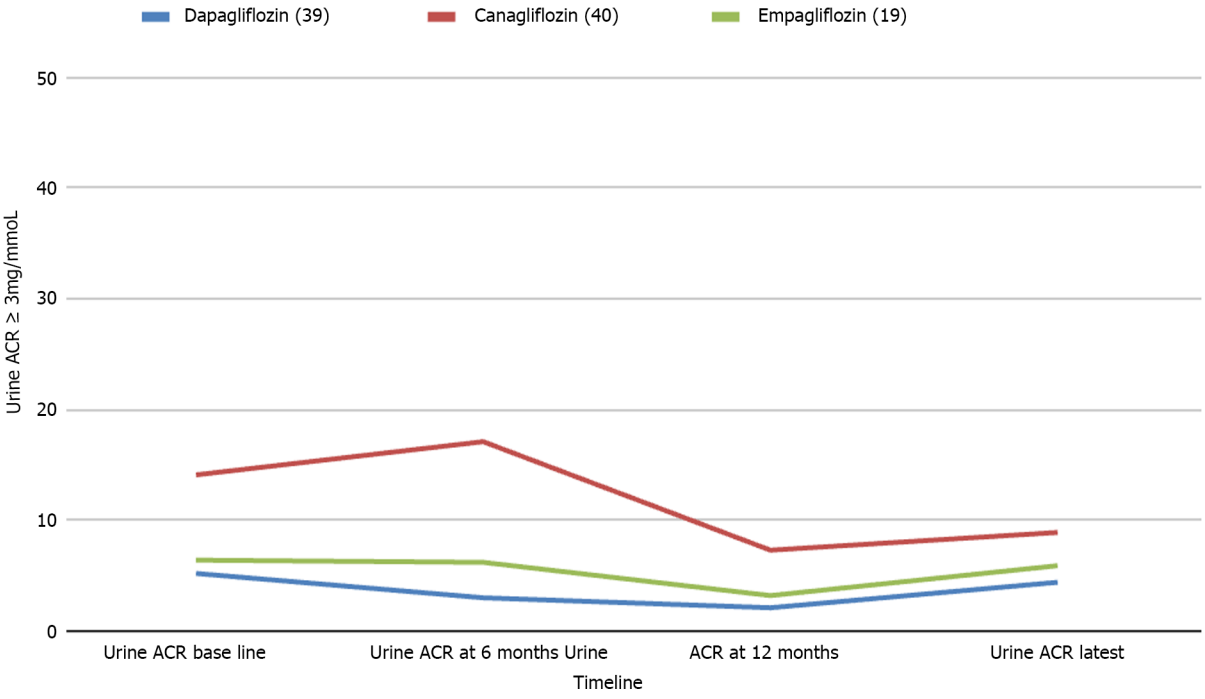

Figure 4 Trends in the urine albumin creatinine ratio among patients with baseline microalbuminuria (urine albumin creatinine ratio ≥ 3 mg/mmol). ACR: Albumin creatinine ratio.

analysis[26]. The tendency for DKA in those with poorer glycemic control observed in the dapagliflozin cohort is highly important in choosing this molecule for managing patients with T2DM. Patients with poor T2DM control are usually insulinopenic and often behave metabolically like those with T1DM[27]. Dapagliflozin use in T1DM patients was associated with a 3.4-fold higher risk of DKA compared to those T2DM patients treated with drug[28]. Therefore, as we observed in our cohort, anyone with a high HbA1c indicating poor glycemic control are at a greater risk of DKA, and clinicians should exercise cation in prescribing dapagliflozin in such patients.

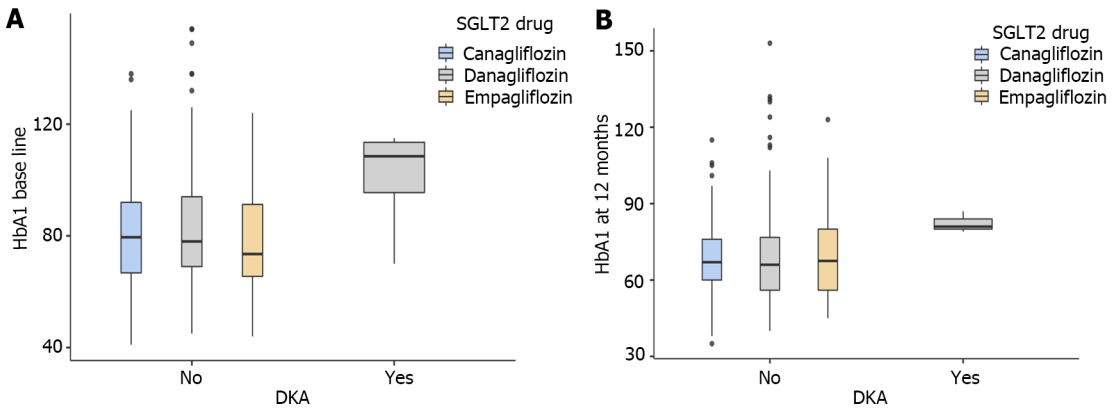

Figure 5 Boxplot analyses of baseline glycated hemoglobin *vs* diabetic ketoacidosis risk in individual sodium glucose cotransporter 2 inhibitor drugs at baseline and after 12 months. A: Box plot explanatory data analysis of baseline glycated hemoglobin (HbA1c) as a risk factor for diabetic ketoacidosis (DKA) with individual sodium glucose cotransporter 2 inhibitor (SGLT2i) drug showing a correlation between the two variables; B: Box plot data analysis of HbA1c level at 12 months as a risk factor for DKA with individual SGLT2i drug showing a correlation between the two variables. DKA: Diabetic ketoacidosis; HbA1c: Glycated hemoglobin; SGLT2: Sodium glucose cotransporter 2.

Canagliflozin

As shown in various studies, canagliflozin use was associated with the maximum HbA1c reduction compared to the other 2 SGLT-2i agents in our study[15,29,30]. However, the median HbA1c reduction was higher than that observed in clinical trials and meta-analyses presumably from the reasons as mentioned above (see dapagliflozin section). Similarly, the weight reduction was also higher than those seen in various studies[15,29-31]. The reason for this also could same as explained above under dapagliflozin section. Interestingly, we found statistically significant reductions in both the systolic and diastolic BP in the canagliflozin group compared to the other 2 molecules.

Canagliflozin use also showed better and consistent reduction in albuminuria compared to the other two SGLT-2i molecules in our study. This could partly be explained by higher baseline ACR in those with albuminuria in the canagliflozin sub-cohort and low numbers of participants in the individual cohorts of the other two drugs in our study (would not have reached significance). Canagliflozin also possesses some inhibitory effect on SGLT-1 receptors which would have potentially helped to improve albuminuria in this cohort as suggested by a recent study[32]. Although ketoacidosis related to SGLT-2i therapy was found to be highest with canagliflozin use compared to other drugs in this class in a recent study[33], we found a lower risk compared to dapagliflozin use in our cohort. Data from more real-world studies might shed light on to the reason for this interesting new observation.

Empagliflozin

Compared to the other two SGLT-2i molecules, the number of participants in the empagliflozin cohort was lower, and therefore some of the outcomes might not have reached statistical significance in the present study. Even then the HbA1c reduction was better for empagliflozin compared to the previous studies[15,29,34], possibly for the same reasons mentioned in the previous sections. For some unexplained reason, the tendency for weight reduction observed in this group at 6 and 12 months of follow up periods did not sustain at the latest follow up. The absence of significant BP reduction, improvement of albuminuria, and DKA in this group may be explained by low numbers in the sub-cohort.

Limitations and strengths of the study

Our study has several limitations as seen in any retrospective data analysis. High attrition rate of cases during follow-up, incomplete data of several of the clinical and biochemical parameters studied, dissimilar numbers of participants in the treatment arms with the three SGLT-2i drugs (downgrading quality of statistical comparisons), and higher baseline HbA1c compared to many previously reported studies, might have reduced the overall quality at least some of the outcome analyses.

Although SGLT-2i drugs have shown to alter lipid parameters with a reduction in triglycerides and improvement of high-density lipoprotein, with a resultant improvement of metabolic syndrome[35], which may be one among the several reasons for their cardiovascular benefits, we could not procure adequate data on lipid parameters (baseline and during follow up period) for inclusion in this study. However, significant improvements in other cardiometabolic parameters like BP, body weight, BMI, and albuminuria make our study results impressive in predicting remarkable future cardiovascular benefits for our cohort.

Moreover, the study results show several unique features such as relatively long period of follow up of our cohort, reasonable record of many of the study parameters and the real-world data with some good comparative and contrasting features with previously reported studies makes our study reasonably robust and should help to inform evidence-based clinical decision-making process by physicians.

CONCLUSION

The real-world use of SGLT-2i medications dapagliflozin, canagliflozin and empagliflozin is associated with significant improvements diabesity, cardiovascular outcomes such as BP reduction and improvement of microalbuminuria, all of which are expected to improve the cardiovascular morbidity and possibly mortality. The adverse effects are relatively low as observed in various clinical trials and meta-analyses. Caution should be exercised while treating T2DM patients with poor glycemic control and high baseline HbA1c with SGLT-2i because of higher risk of developing DKA. Data from larger real-world clinical studies are expected to inform clinical decision making in using SGLT-2i by practitioners more judiciously.

ARTICLE HIGHLIGHTS

Research background

Sodium glucose cotransporter-2 inhibitors (SGLT-2i) are antidiabetic medications with moderate efficacy in glycemic control, and a potential for weight loss through their glycosuric effects, which are helpful for patients with diabesity - diabetes consequent to obesity. Marked cardiovascular and reno-protective effects with the use of SGLT-2i were proven by multiple randomised controlled trials (RCTs), observational studies and various systematic reviews.

Research motivation

Head-to-head comparison of the real-world data on the comparative efficacy and safety of individual SGLT-2i medications is rather sparse and needs more reports to appraise the benefits shown by the above studies.

Research objectives

To procure the comparative efficacy, safety, and adverse effect profiles of SGLT2i drugs in the clinical practice settings to make better therapeutic decision making as RCTs and prospective observational studies are often biased with rigorous monitoring of patients and the study results that wouldn't always reflect the real-world medical practice.

Research methods

We evaluated the comparative efficacy data of 3 SGLT-2i drugs (dapagliflozin, canagliflozin, and empagliflozin) at full doses, used for treating patients with type 2 diabetes mellitus (T2DM) only. Reduction of glycated hemoglobin, body weight, blood pressure, and urine albumin creatinine ratio were recorded, and the adverse effects were documented retrospectively from the clinical records.

Research results

This real-world data from 467 patients with T2DM showed remarkable improvements in diabesity and cardiometabolic outcomes with all the three SGLT-2i agents with a tendency for renal protection (improvement of albuminuria) in those on canagliflozin. These drugs are reasonably safe with acceptably mild side effects profiles when used judiciously in patients with diabesity.

Research conclusions

We found that SGLT-2i class of medications are very useful for the management of diabesity with improvements cardiometabolic outcomes in the real-world settings as proven by previous RCTs and observational studies.

Research perspectives

Management of diabesity is often a clinical dilemma in the context of optimal glycemic control as several antidiabetic agents including insulins tend to cause weight gain with a potential for worsening of diabesity in a vicious cycle. SGLT-2i group of drugs improves glycemic control and cause weight loss when used in patients with diabesity. Moreover, SGLT-2i medications are useful in improving cardiometabolic outcomes and renal protection in patients with T2DM. We studied the comparative efficacy of dapagliflozin, empagliflozin and canagliflozin using a retrospective data from our hospital which revealed significant improvements in body weight, blood pressure, and glycated hemoglobin, with canagliflozin also showing improvement in albuminuria. They were also reasonably safe with acceptable side effect profile when used in the appropriate clinical context.

FOOTNOTES

Co-first authors: Lubna Islam and Dhanya Jose.

Author contributions: Islam L, Alkhalifah M, and Blaibel D collected the clinical data; Jose D performed the statistical analysis and wrote the initial draft of the manuscript; Islam L performed the literature review, and interpretation of relevant data following statistical analysis; Islam L and Jose D helped revision and figure preparation for the paper and share the first authorship of the work; Alkhalifah M, Blaibel D, and Chandrabalan V contributed to the work with additional literature review and revision of the article critically for important intellectual content; Chandrabalan V also procured the patient data from the hospital electronic records; Pappachan JM

contributed to the conceptual design of the paper and critically supervised the whole drafting, literature review, revision and modifications of the paper including figure construction and is the final author; and all authors have read and approved the final version of the manuscript.

Institutional review board statement: The study was approved by the institutional research committee (No: KB/PB/SE-387/2022).

Informed consent statement: This is a retrospective cohort study performed by review of participants' electronic clinical records only and therefore, patient consent was not necessary and was not obtained.

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Dietary fiber intake and its association with diabetic kidney disease in American adults with diabetes: A cross-sectional study

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Abstract

BACKGROUND

Dietary fiber (DF) intake may have a protective effect against type 2 diabetes (T2D); however, its relationship with diabetic kidney disease (DKD) remains unclear.

AIM

To investigate the potential association between DF intake and the prevalence of DKD in individuals diagnosed with T2D.

METHODS

This cross-sectional study used data from the National Health and Nutrition Examination Survey collected between 2005 and 2018. DF intake was assessed through 24-h dietary recall interviews, and DKD diagnosis in individuals with T2D was based on predefined criteria, including albuminuria, impaired glomerular filtration rate, or a combination of both. Logistic regression analysis was used to assess the association between DF intake and DKD, and comprehensive subgroup and sensitivity analyses were performed.

RESULTS

Among the 6032 participants, 38.4% had DKD. With lower DF intake-T1 (≤ 6.4 g/1000 kcal/day)-as a reference, the adjusted odds ratio for DF and DKD for levels T2 (6.5-10.0 g/1000 kcal/day) and T3 (≥ 10.1 g/1000 kcal/day) were 0.97 (95%CI: 0.84-1.12, P = 0.674) and 0.79 (95%CI: 0.68-0.92, P = 0.002), respectively. The subgroup analysis yielded consistent results across various demographic and health-related subgroups, with no statistically significant interactions (all P > 0.05).

CONCLUSION
In United States adults with T2D, increased DF intake may be related to reduced DKD incidence. Further research is required to confirm these findings.

Key Words: Dietary fiber; Diabetic kidney disease; Type 2 diabetes; National Health and Nutrition Examination Survey; Cross-sectional study

Core Tip: This cross-sectional analysis of National Health and Nutrition Examination Survey data, spanning 2005 to 2018, explored the potential correlation between dietary fiber (DF) intake and the prevalence of diabetic kidney disease (DKD) in individuals diagnosed with type 2 diabetes (T2D). The study, which involved 6032 participants, reveals that higher DF intake, particularly in the tertile with ≥ 10.1 g/1000 kcal/day, is associated with a statistically significant reduction in DKD incidence. These findings suggest a potential protective effect of increased DF intake against DKD in adults with T2D in the United States. Further investigation is warranted to corroborate these observations.

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INTRODUCTION

Diabetes is a collective term for metabolic disorders characterized by hyperglycemia stemming from deficiencies in insulin secretion, insulin action, or both[1]. With the continuous expansion of the global economy and improved life expectancy, the prevalence of diabetes has increased. According to data from the International Diabetes Federation Diabetes Atlas, an estimated 783.2 million individuals aged 20-79 years worldwide will be living with diabetes by 2045 [2]. Diabetic kidney disease (DKD) is a complication associated with prolonged diabetes that affects approximately 40% of individuals with type 2 diabetes (T2D) and is one of the most prevalent diabetic complications[3]. DKD markedly increases the risk of cardiovascular events and progression to end-stage renal disease[4,5], culminating in the need for dialysis or renal transplantation. Existing therapeutic approaches offer only symptomatic relief and cannot impede the progression of DKD into chronic kidney disease (CKD)[6]. Therefore, delaying or ameliorating the onset and progression of DKD is crucial.

Dietary fiber (DF) is a composite material composed of indigestible carbohydrates and lignin and resists degradation in the upper gastrointestinal tract. Their primary sources are whole grains, fruits, vegetables, and legumes[7]. Previous studies have highlighted the multifaceted advantages of DF, including its ability to modulate blood glucose[8,9] and lipid levels[9], enhance insulin sensitivity[10], ameliorate inflammatory responses[9], reduce the onset of diabetes[11-13], and lower the prevalence of cardiovascular diseases[14,15] and CKD[16]. However, large-scale studies assessing the association between DF intake and the incidence of DKD in diabetic populations are lacking. Therefore, the current study aimed to investigate whether a higher DF intake reduces the occurrence of DKD in individuals with T2D.

MATERIALS AND METHODS

Study design and participants

The National Health and Nutrition Examination Survey (NHANES) was designed to assess the health and nutritional status of both adults and children in the United States. This program employed a comprehensive approach that combined structured interviews and physical examinations to amass extensive datasets. NHANES operates under the National Center for Health Statistics (NCHS), in partnership with the United States Department of Agriculture, which is responsible for the compilation and dissemination of dietary and nutritional data. Prior to participating in this survey, all NHANES participants provided written informed consent, and the study was approved by the NCHS Institutional Review Board[17]. The survey included intricate measurements, standardized interviews, and laboratory assessments to gather information related to demographics, dietary habits, physical parameters, laboratory diagnostics, and health-related parameters.

This cross-sectional investigation draws from data acquired from NHANES surveys conducted between 2005 and 2018. For more comprehensive details, individuals can access the NHANES resources *via* www.cdc.gov/nchs/nhanes. The current analysis included participants aged 20 years and older who had completed both structured questionnaire surveys and rigorous laboratory examinations. Pregnant women and individuals with missing critical variables were excluded from the analysis.

DF intake

All NHANES participants were eligible to undergo two 24-h dietary recall interviews. The first dietary recall interview took place at the Mobile Examination Center, and the second interview occurred *via* telephone three to ten days later. To ensure the integrity and availability of raw data, data from the first 24-h dietary recall were utilized. Recognizing that total calorie intake may influence dietary data, the data for DF and protein intake used in this study were adjusted for total calorie intake.

Diagnosis of T2D and DKD

The diagnosis of T2D was based on the criteria established by the American Diabetes Association[18] and self-reported questionnaires. Participants were classified as having T2D if they met any of the following conditions: (1) A documented previous diagnosis by a medical practitioner; (2) current utilization of insulin or oral hypoglycemic medications; (3) fasting plasma glucose (FPG) ≥ 7.0 mmol/L; (4) glycosylated hemoglobin (HbA1c) ≥ 6.5%; and or (5) plasma glucose ≥ 11.1 mmol/L 2 h post oral glucose tolerance test. The diagnosis of DKD in patients with T2D was established when the albumin-to-creatinine ratio (ACR) exceeded 30 mg/g and/or the estimated glomerular filtration rate (eGFR) fell below 60 mL/min/1.73 m^2[19]. ACR was computed by assessing the urine albumin-to-urine creatinine ratio, whereas eGFR was determined using the CKD Epidemiology Collaboration algorithm[20].

Other potential variables

The current study examined variables that could influence the association between DF intake and DKD. These variables included age, sex, race/ethnicity, educational level, marital status, family income, body mass index (BMI), smoking status, daily calorie and protein intake, dietary supplement usage, history of coronary heart disease (CHD), hypertension, family history of diabetes, age at initial diabetes diagnosis, insulin and glucose-lowering drug utilization, blood pressure, and various laboratory tests including HbA1c, fasting plasma insulin (FINS), FPG, total cholesterol (TC), alanine aminotransferase (ALT), aspartate aminotransferase, serum uric acid (SUA), blood urea nitrogen, serum creatinine (sCr), HbA1c, urine albumin, and urine creatinine. Race/ethnicity was categorized into non-Hispanic white, non-Hispanic black, Mexican American, or other ethnic groups. Educational level was stratified by the number of years of completed education as follows: < 9 years, 9-12 years, and > 12 years. Marital status was classified as being married, living with a partner, or living alone, based on questionnaire responses. Family income was determined using the family poverty income ratio and categorized as low (≤ 1.3), medium (1.3–3.5), and high (> 3.5) income levels[17]. Smoking status was divided into three categories: Never smoked, former smoker, and current smoker. Never-smokers were individuals who had smoked < 100 cigarettes in their lifetime. Former smokers were defined as those who had smoked more than 100 cigarettes in their lifetime but were not currently smoking. Current smokers were defined as individuals who had smoked at least 100 cigarettes in their lifetime and were currently smoking. The use of dietary supplements was defined as the use of dietary supplements or medications in the past month. The diagnosis of CHD was ascertained through a questionnaire item inquiring about prior diagnosis of CHD. The diagnosis of hypertension included systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg, self-reported diagnosis with hypertension, or being informed about hypertension on two or more occasions. A family history of diabetes was determined through questions concerning close relatives with diabetes. The duration of diabetes was calculated by subtracting the age at initial diagnosis with diabetes from the age t time of the interview. Those diagnosed within the last year were categorized as having diabetes for half a year. The use of insulin- and glucose-lowering drugs was determined based on specific questionnaire responses. BMI was calculated using height and weight measurements. The homeostasis model assessment of insulin resistance (HOMA-IR), employed to evaluate insulin resistance, was calculated using the formula HOMA-IR = FINS (μU/mL) × FPG (mmol/L)/22.5[21]. Further details regarding the measurement techniques used for these variables are available at www.cdc.gov/nchs/nhanes/.

Statistical analysis

Data analyses were conducted using R Statistical Software (http://www.R-project.org, The R Foundation) and the Free Statistics analysis platform (Beijing, China). Continuous variables are presented as means ± SD or medians (interquartile range), while categorical variables are reported as frequencies or percentages. To compare baseline characteristics between groups based on DF intake, independent t-tests were used for continuous variables, and chi-square tests were used for categorical variables. Univariate logistic regression analysis was performed to explore the relationship between the potential factors and DKD. Subsequently, a multivariate logistic regression analysis was performed to ascertain the association between DF intake and DKD. Model 1 was adjusted for age, sex, race/ethnicity, educational level, marital status, and family income. Model 2 incorporated all the covariates from Model 1 and included additional adjustments for smoking status, CHD, hypertension, and daily calorie and protein consumption. Model 3 extended the adjustments to encompass the duration of diabetes; insulin usage; and HbA1c, HOMA-IR, TC, ALT, SUA, and HbA1c levels. To explore the potential nonlinear relationship between DF intake and DKD, restricted cubic splines (RCS) were applied while controlling for all covariates in the full model (Model 3). Subgroup analyses were conducted by stratifying the data into five subgroups based on age (20-60 years *vs* ≥ 60 years), sex (male *vs* female), BMI (< 25 kg/m^2 *vs* ≥ 25 kg/m^2), HbA1c level (< 7.0% *vs* ≥ 7.0%), and eGFR level (< 90 mL/min/1.73 m^2 *vs* ≥ 90 mL/min/1.73 m^2). Additionally, this study explored the interactions between DF intake and these factors. To ensure the robustness of the findings, the relationship between DF intake and DKD was re-examined after excluding extreme energy intake values (< 500 kcal/day or > 5000 kcal/day). To address missing data, multiple imputation techniques were employed to fill existing gaps and facilitate a comprehensive analysis.

RESULTS

Study population

This study included 70190 participants from NHANES 2005-2018, of whom 39749 were aged 20 years or older. However, the study excluded pregnant women (n = 708), individuals with missing ACR and eGFR data (n = 4476), those lacking dietary data (n = 2150), and those who did not meet the diagnostic criteria for T2D (n = 26383). This resulted in a final sample size of 6032 participants, of which 2316 were diagnosed with DKD. The inclusion and exclusion criteria are shown in Figure 1.

Baseline characteristics

Table 1 presents the baseline characteristics of all the participants categorized into tertiles based on their DF intake. In total, 2316 (38.4%) patients had DKD. The average age of the study participants was 61.1 years, and 2892 (47.9%) were female. Individuals who consumed more DF tended to be older, female, Mexican American, married, living with a partner, or a never smoker, or have a lower educational level, lower BMI, lower daily calorie consumption, and higher protein consumption. Moreover, this group was more likely to use dietary supplements and glucose-lowering drugs and had a longer duration of T2D. Notably, they displayed lower levels of FINS, HOMA-IR, TC, SUA, sCr, HbA1c, and diastolic blood pressure.

Relationship between DF intake and DKD

Univariate analysis revealed that age; race/ethnicity; educational level; marital status; family income; smoking status; CHD; hypertension; daily calorie and DF consumption; duration of diabetes; insulin usage; and levels of HbA1c, FPG, HOMA-IR, TC, ALT, SUA, and HbA1c were associated with DKD (Table 2).

To ascertain the independent association between DF intake and DKD, multivariate analyses were performed with adjustments in the three models. As indicated in Table 3, DF intake and DKD were inversely related, with an adjusted OR value of 0.97 (95%CI: 0.96-0.98, $P < 0.001$). Compared with individuals with a lower DF intake belonging to Tertile 1 (T1, ≤ 6.4 g/1000 kcal/day), the unadjusted OR values for DF intake and DKD in Tertile 2 (T2, 6.5-10.0 g/1000 kcal/day) and Tertile 3 (T3, ≥ 10.1 g/1000 kcal/day) were 0.97 (95%CI: 0.84-1.12, $P = 0.674$) and 0.79 (95%CI: 0.68-0.92, $P = 0.002$), respectively. Accordingly, the relationship between DF intake and DKD exhibited an inverse linear pattern (nonlinear, $P = 0.814$) in RCS analysis (Supplementary Figure 1).

Subgroup analysis

The association between DF intake and DKD was consistent across multiple subgroups. As illustrated in Figure 2, no significant interaction was detected in any subgroup after stratification by age, sex, BMI, HbA1c level, or eGFR ($P > 0.05$).

Sensitivity analysis

After excluding individuals with extreme energy intake, 5910 individuals remained, and the association between DF intake and DKD was stable. Compared with individuals with a lower DF intake, T1 (≤ 6.4 g/1000 kcal/day), the unadjusted OR values for DF intake and DKD in T2 (6.5-10.0 g/1000 kcal/day), and T3 (≥ 10.1 g/1000 kcal/day) were 1.00 (95%CI: 0.86-1.16, $P = 0.996$), and 0.82 (95%CI: 0.70-0.96, $P = 0.011$), respectively (Supplementary Table 1).

DISCUSSION

This cross-sectional study of adults with T2D in the United States revealed an inverse correlation between DF intake and the occurrence of DKD. Higher intake of DF may be associated with a reduced incidence of DKD. Subgroup and sensitivity analyses further reinforced the stability of these findings.

Numerous studies have highlighted the potential benefits of increasing DF intake in reducing the incidence of diabetes. A seven-year prospective study discovered that individuals with lower total DF intake were more susceptible to developing diabetes[11]. Additionally, an analysis of multiple prospective cohort studies supported the association between increased daily intake of cereal fiber and decreased risk of T2D[12]. The potential protective effects of DF intake against diabetes may be linked to its ability to enhance blood sugar control, regulate lipid levels, manage body weight, and modulate inflammatory responses[9]. These effects were also observed in the present study. The high DF intake group exhibited lower FINS, HOMA-IR, TC, and BMI.

Research indicates that the protective effects of cereal fibers against diabetes may be attributed to their modulation of the gut microbiota through various mechanisms, including the enhancement of glucose tolerance, reduction of inflammation, and alteration of immune responses *via* energy metabolism pathways such as colonic fermentation and production of short-chain fatty acids (SCFAs)[22]. DF serves as the primary energy source for most gut microbiota and undergoes fermentation in the intestine, resulting in the production of active intestinal metabolites called SCFAs such as butyrate, propionate, and acetate[23]. Studies have revealed a deficiency in SCFAs, particularly butyrate, in the gut of individuals with T2D[24]. This insufficiency is predominantly ascribed to alterations in the composition of the gut microbiota, resulting in diminished production of SCFAs[25]. In a randomized controlled trial, Zhao *et al*[24] identified a distinct cohort of SCFA producers that contributed to the amelioration of T2D through augmented SCFA production. Notably, an increase in DF intake can modulate the generation of SCFA metabolites[24]. Inflammation and fibrosis play pivotal roles in the pathophysiology of DKD. Significantly, discernible differences exist in the abundance and

Table 1 Population characteristics by categories of dietary fiber intake

Variables	Total	Dietary fiber intake, g/1000 kcal/d			*P* value
		T1 (≤ 6.4)	T2 (6.5-10.0)	T3 (≥ 10.1)	
N	6032	1978	2000	2054	
Age (yr), mean (SD)	61.1 (13.5)	58.3 (14.2)	61.8 (13.3)	63.0 (12.6)	< 0.001
Sex, *n* (%)					< 0.001
Male	3140 (52.1)	1146 (57.9)	1011 (50.5)	983 (47.9)	
Female	2892 (47.9)	832 (42.1)	989 (49.5)	1071 (52.1)	
Race/ethnicity, *n* (%)					< 0.001
Non-Hispanic white	2210 (36.6)	799 (40.4)	789 (39.5)	622 (30.3)	
Non-Hispanic black	1481 (24.6)	626 (31.6)	465 (23.2)	390 (19.0)	
Mexican American	1126 (18.7)	237 (12.0)	355 (17.8)	534 (26.0)	
Others	1215 (20.1)	316 (16.0)	391 (19.6)	508 (24.7)	
Educational level (yr), *n* (%)					< 0.001
< 9	1067 (17.7)	265 (13.4)	288 (14.4)	514 (25.0)	
9-12	2406 (39.9)	863 (43.6)	801 (40.1)	742 (36.1)	
> 12	2559 (42.4)	850 (43.0)	911 (45.6)	798 (38.9)	
Marital status, *n* (%)					0.013
Married or living with a partner	3649 (60.5)	1164 (58.8)	1190 (59.5)	1295 (63.0)	
Living alone	2383 (39.5)	814 (41.2)	810 (40.5)	759 (37.0)	
Family income, *n* (%)					0.340
Low	2151 (35.7)	706 (35.7)	682 (34.1)	763 (37.1)	
Medium	2453 (40.7)	795 (40.2)	836 (41.8)	822 (40.0)	
High	1428 (23.7)	477 (24.1)	482 (24.1)	469 (22.8)	
BMI (kg/m^2), mean (SD)	32.3 (7.4)	33.1 (7.9)	32.5 (7.3)	31.5 (6.8)	< 0.001
Smoking status, *n* (%)					< 0.001
Never	3029 (50.2)	893 (45.1)	952 (47.6)	1184 (57.6)	
Former	2032 (33.7)	601 (30.4)	749 (37.5)	682 (33.2)	
Current	971 (16.1)	484 (24.5)	299 (14.9)	188 (9.2)	
CHD, *n* (%)	607 (10.1)	192 (9.7)	206 (10.3)	209 (10.2)	0.807
Hypertension, *n* (%)	3942 (65.4)	1281 (64.8)	1316 (65.8)	1345 (65.5)	0.780
Calorie consumption (1000 kcal/d), mean (SD)	1.9 (0.9)	2.1 (1.0)	1.9 (0.9)	1.7 (0.7)	< 0.001
Protein consumption (g/1000 kcal/d), mean (SD)	41.2 (13.3)	40.4 (14.7)	40.9 (12.6)	42.2 (12.5)	< 0.001
Dietary fiber (g/1000 kcal/d), median (IQR)	8.2 (5.7, 11.4)	4.7 (3.7, 5.7)	8.1 (7.3, 9.0)	13.1 (11.3, 16.1)	< 0.001
Dietary supplements taken, *n* (%)	3300 (54.7)	981 (49.6)	1105 (55.2)	1214 (59.1)	< 0.001
Family history of diabetes, *n* (%)	3840 (63.7)	1276 (64.5)	1265 (63.2)	1299 (63.2)	0.632
Duration of diabetes (yr), median (IQR)	8.0 (3.0, 15.0)	8.0 (3.0, 15.0)	9.0 (3.0, 15.0)	9.0 (4.0, 16.0)	< 0.001
Insulin use, *n* (%)	1160 (19.2)	384 (19.4)	378 (18.9)	398 (19.4)	0.900
Glucose-lowering drugs use, *n* (%)	4092 (67.8)	1237 (62.5)	1393 (69.7)	1462 (71.2)	< 0.001
HbA1c (%), mean (SD)	7.2 (1.7)	7.3 (1.9)	7.2 (1.7)	7.2 (1.7)	0.404

FPG (mmol/L), mean (SD)	8.6 (3.4)	8.7 (3.6)	8.6 (3.2)	8.5 (3.3)	0.088
FINS (μU/mL), median (IQR)	14.1 (8.5, 23.9)	15.3 (8.9, 26.1)	14.3 (8.5, 23.9)	13.3 (8.2, 21.9)	< 0.001
HOMA-IR, median (IQR)	5.1 (2.8, 8.9)	5.4 (3.0, 10.1)	5.1 (2.9, 8.9)	4.8 (2.7, 8.1)	< 0.001
TC (mmol/L), mean (SD)	4.8 (1.2)	4.9 (1.3)	4.8 (1.2)	4.8 (1.2)	< 0.001
ALT (U/L), median (IQR)	22.0 (16.0, 30.0)	22.0 (16.0, 31.0)	21.0 (16.0, 29.0)	22.0 (17.0, 29.0)	0.191
AST (U/L), median (IQR)	23.0 (19.0, 28.0)	23.0 (19.0, 28.0)	23.0 (19.0, 28.0)	23.0 (19.0, 28.0)	0.231
SUA (μmol/L), mean (SD)	344.4 (94.4)	352.5 (99.4)	343.4 (92.2)	337.6 (90.9)	< 0.001
BUN (mmol/L), median (IQR)	5.0 (3.9, 6.8)	5.0 (3.9, 6.4)	5.4 (3.9, 6.8)	5.4 (3.9, 6.8)	< 0.001
sCr (μmol/L), median (IQR)	79.6 (63.6, 97.2)	80.4 (67.2, 99.0)	79.6 (64.5, 97.2)	76.0 (61.9, 95.5)	< 0.001
ACR (mg/g), median (IQR)	12.9 (6.8, 40.6)	12.5 (6.7, 43.5)	13.1 (6.8, 43.8)	12.9 (6.8, 35.9)	0.487
eGFR (ml/min/1.73 m^2), mean (SD)	83.3 (24.3)	83.9 (25.0)	82.4 (24.1)	83.7 (23.9)	0.103
Hemoglobin (g/dL), mean (SD)	13.9 (1.6)	14.0 (1.7)	13.8 (1.6)	13.7 (1.6)	< 0.001
Systolic blood pressure (mmHg), mean (SD)	132.0 (20.1)	131.4 (19.8)	132.0 (20.1)	132.7 (20.4)	0.124
Diastolic blood pressure (mmHg), mean (SD)	69.3 (13.5)	70.9 (13.5)	69.3 (13.5)	68.0 (13.2)	< 0.001
DKD, *n* (%)	2316 (38.4)	765 (38.7)	803 (40.2)	748 (36.4)	0.048

ACR: Albumin creatinine ratio; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; BUN: Blood urea nitrogen; CHD: Coronary heart disease; DKD: Diabetic kidney disease; eGFR: Estimated glomerular filtration rate; FINS: Fasting plasma insulin; FPG: Fasting plasma glucose; HbA1c: Glycosylated hemoglobin; HOMA-IR: Homeostasis model assessment of insulin resistance; sCr: Serum creatinine; SUA: Serum uric acid; TC: Total cholesterol.

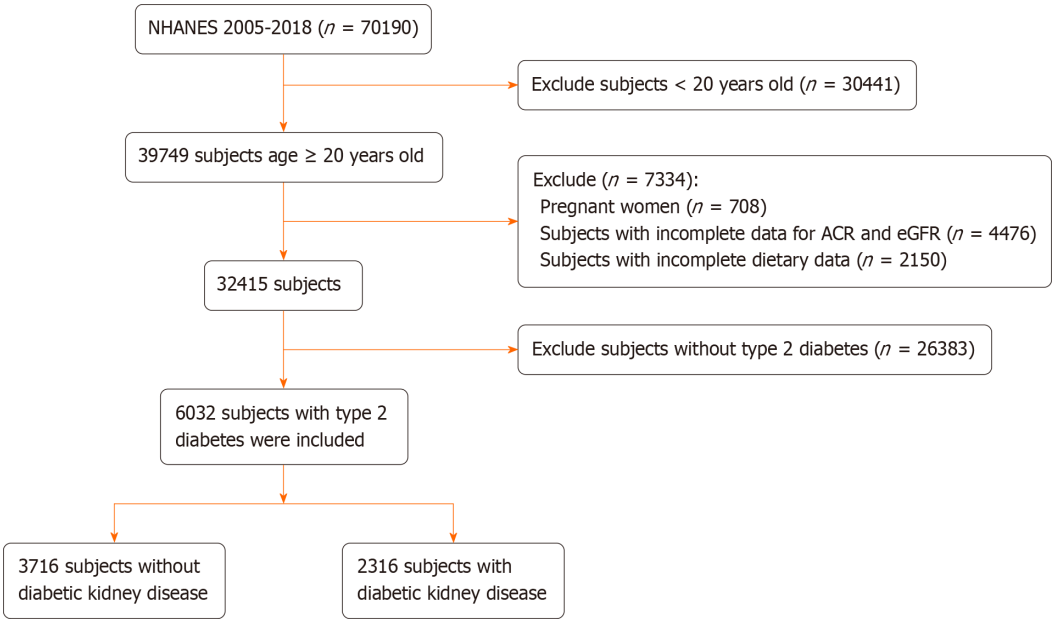

Figure 1 Flow diagram of the study. NHANES: National Health and Nutrition Examination Survey; ACR: Albumin-to-creatinine; eGFR: Estimated glomerular filtration rate.

composition of the gut microbiota between individuals with DKD and healthy controls[26]. Research suggests that the gut microbiota may affect DKD development by modulating endocrine functions and the composition of microbial metabolic byproducts within the gut[27]. Notably, SCFAs derived from the gut microbiota can regulate inflammation, oxidative stress, fibrosis, and energy metabolism, thus offering preventive and therapeutic potential in DKD[6]. Experimental evidence indicates a notable decrease in DKD incidence in diabetes-induced animals fed a high-fiber diet, emphasizing the potential renoprotective role of DF. This protective effect is attributed to its capacity to reshape the gut microbiota ecology, mitigate dysbiosis, and stimulate the proliferation of SCFA-producing bacteria. Consequently, SCFA concentrations increase, exerting a protective effect against DKD through the GPR43 and GPR109A pathways[28,29].

Table 2 Association of covariates and diabetic kidney disease risk

Variables	OR (95%CI)	*P* value
Age (yr)	1.04 (1.04-1.05)	< 0.001
Sex, *n* (%)		
Male	1 (reference)	
Female	0.93 (0.84-1.03)	0.178
Race/ethnicity, *n* (%)		
Non-Hispanic white	1 (reference)	
Non-Hispanic black	1.11 (0.97-1.27)	0.115
Mexican American	0.80 (0.69-0.93)	0.003
Others	0.72 (0.62-0.83)	< 0.001
Educational level (yr), *n* (%)		
< 9	1 (reference)	
9-12	0.97 (0.84-1.12)	0.651
> 12	0.73 (0.63-0.85)	< 0.001
Marital status, *n* (%)		
Married or living with a partner	1 (reference)	
Living alone	1.45 (1.31-1.61)	< 0.001
Family income, *n* (%)		
Low	1 (reference)	
Medium	0.96 (0.85-1.08)	0.466
High	0.62 (0.54-0.72)	< 0.001
BMI (kg/m^2)	1.00 (0.99-1.01)	0.605
Smoking status, *n* (%)		
Never	1 (reference)	
Former	1.34 (1.20-1.51)	< 0.001
Current	1.08 (0.93-1.25)	0.328
CHD, *n* (%)		
No	1 (reference)	
Yes	2.16 (1.82-2.56)	< 0.001
Hypertension, *n* (%)		
No	1 (reference)	
Yes	2.24 (2.00-2.52)	< 0.001
Calorie consumption (1000 kcal/d)	0.83 (0.78-0.89)	< 0.001
Protein consumption (g/1000 kcal/d)	1.00 (1.00-1.00)	0.965
Dietary fiber consumption (g/1000 kcal/d)	0.98 (0.97-0.99)	< 0.001
Dietary supplements taken, *n* (%)		
No	1 (reference)	
Yes	1.06 (0.95-1.17)	0.313
Family history of diabetes, *n* (%)		
No	1 (reference)	
Yes	1.04 (0.93-1.16)	0.487
Duration of diabetes (yr)	1.04 (1.03-1.04)	< 0.001

Insulin use, *n* (%)		
No	1 (reference)	
Yes	2.32 (2.03-2.64)	< 0.001
Glucose-lowering drugs use, *n* (%)		
No	1 (reference)	
Yes	1.03 (0.92-1.15)	0.576
HbA1c (%)	1.15 (1.11-1.18)	< 0.001
FPG (mmol/L)	1.06 (1.04-1.07)	< 0.001
FINS (μU/mL)	1.00 (1.00-1.00)	0.132
HOMA-IR	1.01 (1.00-1.01)	< 0.001
TC (mmol/L)	0.95 (0.91-0.99)	0.012
ALT (U/L)	1.00 (0.99-1.00)	0.019
AST (U/L)	1.00 (1.00-1.00)	0.816
SUA (μmol/L)	1.00 (1.00-1.01)	< 0.001
Hemoglobin (g/dL)	0.81 (0.79-0.84)	< 0.001

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; CHD: Coronary heart disease; FINS: Fasting plasma insulin; FPG: Fasting plasma glucose; HbA1c: Glycosylated hemoglobin; HOMA-IR: Homeostasis model assessment of insulin resistance; SUA: Serum uric acid; TC: Total cholesterol.

Table 3 Association between dietary fiber and diabetic kidney disease

Variable	Total, *n*	Events, *n* (%)	Model 1		Model 2		Model 3	
			OR (95%CI)	*P* value	OR (95%CI)	*P* Value	OR (95%CI)	*P* value
DF intake (g/1000 kcal/d)	6032	2316 (38.4)	0.97 (0.96-0.98)	< 0.001	0.97 (0.96-0.98)	< 0.001	0.97 (0.96-0.98)	< 0.001
Tertile groups (g/1000 kcal/d)								
T1 (≤ 6.4)	1978	765 (38.7)	1 (reference)		1 (reference)		1 (reference)	
T2 (6.5-10.0)	2000	803 (40.2)	0.96 (0.84-1.10)	0.544	0.97 (0.84-1.11)	0.642	0.97 (0.84-1.12)	0.674
T3 (≥ 10.1)	2054	748 (36.4)	0.78 (0.68-0.90)	0.001	0.79 (0.68-0.91)	0.001	0.79 (0.68-0.92)	0.002
P for trend				< 0.001		0.001		0.002

Model 1 was adjusted for age, sex, race/ethnicity, educational level, marital status, and family income. Model 2 was adjusted for all covariates in Model 1 in addition to smoking status, coronary heart disease, hypertension, calorie consumption, and protein consumption. Model 3 was adjusted for all covariates in Model 2 plus the duration of diabetes, insulin use, and glycosylated hemoglobin, homeostasis model assessment of insulin resistance, total cholesterol, alanine aminotransferase, serum uric acid, hemoglobin levels. DF: Dietary fiber.

Simultaneously, *in vitro* experiments have suggested that SCFAs modulate inflammatory responses in renal tubular cells and podocytes under hyperglycemic conditions[29]. Butyrate, a SCFA, may alleviate renal inflammation and fibrosis in mice through various pathways[30,31], thereby mitigating DKD. Furthermore, research indicates that SCFAs enhance autophagic capability in renal tubular cells of diabetic mice through the histone deacetylase/unc-51 Like autophagy-activating kinase 1 axis, thus alleviating renal fibrosis[32]. The correlation between the gastrointestinal system and the kidneys is called the "gut-kidney axis"[33]. These studies underscore the significance of gut microbiota in DKD, highlighting the interplay between DF, gut microbiota, SCFAs, and renal health.

To the best of our knowledge, this is the first study to investigate the correlation between DF intake and DKD in a large cohort of patients with T2D. However, the current study had certain limitations. First, akin to most cross-sectional investigations, it can only delineate an association between DF consumption and DKD but cannot establish causation. Second, the data on DF intake relied on 24-hour dietary recall information from the NHANES, which may be susceptible to recall bias. Third, distinct sources of DF may have different effects. Unfortunately, specific data about the sources of DF consumption in NHANES were not obtained. Future studies should consider including these factors. Finally, the current investigation focused on the adult population with diabetes in the United States. To address this limitation, we aim to

Subgroup	Variable	Total	Event (%)	OR (95%CI)	*P* for interation
Age, years					
20–60					0.347
	T1	952	259 (27.2)	1(reference)	
	T2	756	210 (27.8)	1.02 (0.80~1.28)	
	T3	659	139 (21.1)	0.70 (0.54~0.91)	
≥ 60					
	T1	1026	506 (49.3)	1(reference)	
	T2	1244	593 (47.7)	0.96 (0.80~1.15)	
	T3	1395	609 (43.7)	0.81 (0.68~0.98)	
Sex					
Male					0.529
	T1	1146	462 (40.3)	1(reference)	
	T2	1011	415 (41.0)	0.94 (0.78~1.13)	
	T3	983	354 (36.0)	0.73 (0.60~0.89)	
Female					
	T1	832	303 (36.4)	1(reference)	
	T2	989	388 (39.2)	1.03 (0.83~1.28)	
	T3	1071	394 (36.8)	0.88 (0.70~1.10)	
BMI, kg/m^2					
< 25.0					0.995
	T1	245	100 (40.8)	1(reference)	
	T2	244	109 (44.7)	1.00 (0.64~1.55)	
	T3	311	120 (38.6)	0.79 (0.51~1.23)	
≥ 25.0					
	T1	1733	665 (38.4)	1(reference)	
	T2	1756	694 (39.5)	0.97 (0.84~1.13)	
	T3	1743	628 (36.0)	0.80 (0.68~0.94)	
HbA1c, %					
< 7.0					0.46
	T1	1147	399 (34.8)	1(reference)	
	T2	1154	416 (36.0)	0.94 (0.78~1.14)	
	T3	1182	379 (32.1)	0.73 (0.60~0.89)	
≥ 7.0					
	T1	831	366 (44.0)	1(reference)	
	T2	846	387 (45.7)	1.03 (0.84~1.28)	
	T3	872	369 (42.3)	0.89 (0.72~1.11)	

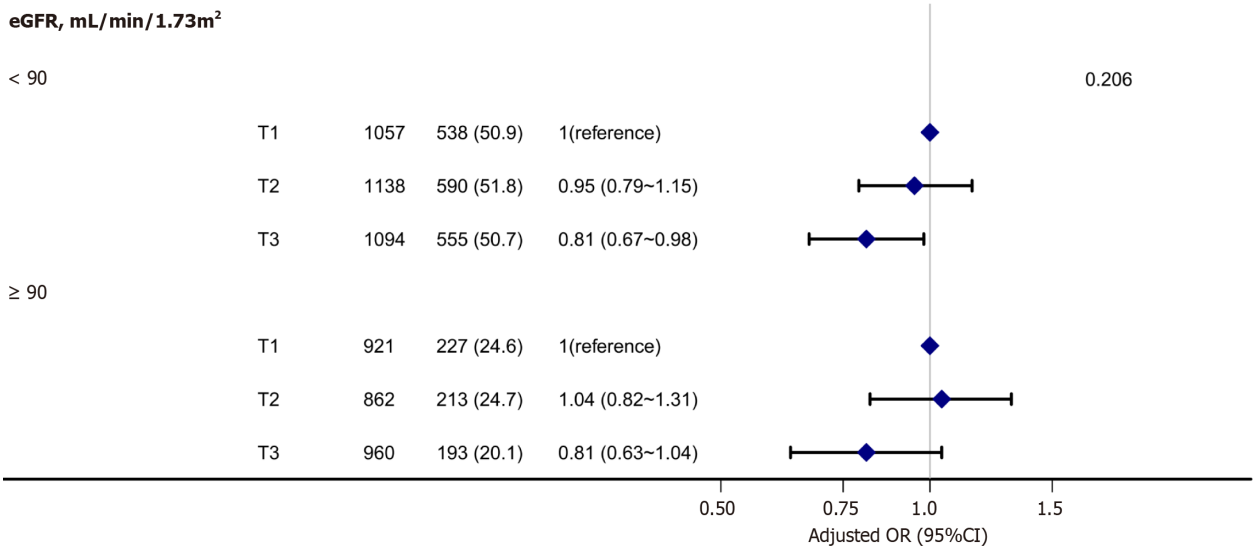

Figure 2 Relationship between dietary fiber intake and diabetic kidney disease according to basic features. Except for the stratification component itself, each stratification factor is adjusted for all other variables (age, sex, race/ethnicity, educational level, marital status, family income, smoking status, coronary heart disease, hypertension, calorie consumption, protein consumption, duration of diabetes, insulin use, and hemoglobin, homeostasis model assessment of insulin resistance, total cholesterol, alanine aminotransferase, serum uric acid, and hemoglobin levels). T1: ≤ 6.4 g/1000 kcal/day; T2: 6.5-10.0 g/1000 kcal/day; T3: ≥ 10.1 g/1000 kcal/day; eGFR: Estimated glomerular filtration rate; BMI: Body mass index; HbA1c: Hemoglobin.

validate current findings in future studies that include a more diverse population.

CONCLUSION

In an adult population with T2D in the United States, elevated DF intake may be associated with a decreased incidence of DKD. However, further research is required to confirm this hypothesis.

ARTICLE HIGHLIGHTS

Research background

This study focused on the relationship between dietary fiber (DF) intake and diabetic kidney disease (DKD) in individuals with type 2 diabetes (T2D). The prevalence of T2D is increasing globally. The protective effect of DF against T2D is acknowledged, but its specific impact on DKD remains unclear. DKD poses a substantial health burden, underscoring the importance of investigating modifiable factors such as DF intake for potential preventive strategies.

Research motivation

The main topics driving this research included understanding the potential protective role of DF against DKD, a complication frequently associated with T2D. Key problems to be addressed include the lack of conclusive evidence on the DF-DKD relationship and the need for targeted interventions to mitigate DKD risk in T2D patients. Solving these problems is crucial for future research on diabetes management and the prevention of kidney disease.

Research objectives

The primary objective of this study was to investigate the association between DF intake and the prevalence of DKD in T2D individuals. Realizing these objectives contributes to filling gaps in the current knowledge regarding the role of DF in DKD prevention, providing insights for future research to refine dietary recommendations for individuals with T2D.

Research methods

This study employed a cross-sectional design utilizing National Health and Nutrition Examination Survey data collected between 2005 and 2018. DF intake was assessed through 24-h dietary recall interviews, and DKD diagnosis was based on predefined criteria, including albuminuria and impaired glomerular filtration rate. This study employed various statistical methods including multiple regression models, restricted cubic splines, stratified analysis with interactions, and sensitivity analysis.

Research results

Of the 6032 participants, 38.4% presented with DKD. The study reveals a significant association between higher DF intake and reduced odds of DKD, particularly in the highest intake tier (T3: ≥ 10.1 g/1000 kcal/day).

Research conclusions

This study proposes that an increased DF intake is associated with a reduced incidence of DKD in adults with T2D. These findings contribute to the field by suggesting potentially modifiable factors for DKD prevention of T2D in individuals.

Research perspectives

Future research should delve deeper into the mechanisms underlying this observed association and explore the feasibility of dietary interventions to prevent or manage DKD in T2D patients. Additionally, longitudinal studies are warranted to establish causality and inform evidence-based dietary guidelines for individuals with T2D at risk of DKD.

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FOOTNOTES

Author contributions: Jia XH made significant contributions to research design, data collection, analysis, manuscript writing, and revision; Wang SY contributed to research design and data collection; Sun AQ played a pivotal role in data analysis; all authors have read and approve the final manuscript.

Institutional review board statement: Ethical review and approval were waived for this study because no additional institutional review board approval was required for the secondary analysis.

Informed consent statement: The data for this study were sourced from the National Center for Health Statistics (NCHS) database. The National Health and Nutrition Examination Survey received authorization from the Ethics Review Committee of the NCHS, and all participants duly completed written informed consent forms prior to their engagement.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: Publicly accessible datasets pertinent to this study are accessible online. The nomenclature of the repository/repositories can be found at the following web address: http://www.cdc.gov/nchs/nhanes.htm.

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ORIGINAL ARTICLE

Basic Study

MicroRNA-630 alleviates inflammatory reactions in rats with diabetic kidney disease by targeting toll-like receptor 4

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Abstract

BACKGROUND

Diabetic kidney disease (DKD) is a major complication of diabetes mellitus. Renal tubular epithelial cell (TEC) damage, which is strongly associated with the inflammatory response and mesenchymal trans-differentiation, plays a significant role in DKD; However, the precise molecular mechanism is unknown. The recently identified microRNA-630 (miR-630) has been hypothesized to be closely associated with cell migration, apoptosis, and autophagy. However, the association between miR-630 and DKD and the underlying mechanism remain unknown.

AIM

To investigate how miR-630 affects TEC injury and the inflammatory response in DKD rats.

METHODS

Streptozotocin was administered to six-week-old male rats to create a hypergly-

cemic diabetic model. In the second week of modeling, the rats were divided into control, DKD, negative control of lentivirus, and miR-630 overexpression groups. After 8 wk, urine and blood samples were collected for the kidney injury assays, and renal tissues were removed for further molecular assays. The target gene for miR-630 was predicted using bioinformatics, and the association between miR-630 and toll-like receptor 4 (TLR4) was confirmed using *in vitro* investigations and double luciferase reporter gene assays. Overexpression of miR-630 in DKD rats led to changes in body weight, renal weight index, basic blood parameters and histopathological changes.

RESULTS

The expression level of miR-630 was reduced in the kidney tissue of rats with DKD ($P < 0.05$). The miR-630 and TLR4 expressions in rat renal TECs (NRK-52E) were measured using quantitative reverse transcription polymerase chain reaction. The mRNA expression level of miR-630 was significantly lower in the high-glucose (HG) and HG + mimic negative control (NC) groups than in the normal glucose (NG) group ($P < 0.05$). In contrast, the mRNA expression level of TLR4 was significantly higher in these groups ($P < 0.05$). However, miR-630 mRNA expression increased and TLR4 mRNA expression significantly decreased in the HG + miR-630 mimic group than in the HG + mimic NC group ($P < 0.05$). Furthermore, the levels of tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and IL-6 were significantly higher in the HG and HG + mimic NC groups than in NG group ($P < 0.05$). However, the levels of these cytokines were significantly lower in the HG + miR-630 mimic group than in the HG + mimic NC group ($P < 0.05$). Notably, changes in protein expression were observed. The HG and HG + mimic NC groups showed a significant decrease in E-cadherin protein expression, whereas TLR4, α-smooth muscle actin (SMA), and collagen IV protein expression increased ($P < 0.05$). Conversely, the HG + miR-630 mimic group exhibited a significant increase in E-cadherin protein expression and a notable decrease in TLR4, α-SMA, and collagen IV protein expression than in the HG + mimic NC group ($P < 0.05$). The miR-630 targets TLR4 gene expression. In vivo experiments demonstrated that DKD rats treated with miR-630 agomir exhibited significantly higher miR-630 mRNA expression than DKD rats injected with agomir NC. Additionally, rats treated with miR-630 agomir showed significant reductions in urinary albumin, blood glucose, TLR4, and proinflammatory markers (TNF-α, IL-1β, and IL-6) expression levels ($P < 0.05$). Moreover, these rats exhibited fewer kidney lesions and reduced infiltration of inflammatory cells.

CONCLUSION

MiR-630 may inhibit the inflammatory reaction of DKD by targeting TLR4, and has a protective effect on DKD.

Key Words: Diabetic kidney disease; MicroRNA-630; Toll-like receptor 4; Mouse model; Renal tubular epithelial cells damage; Hyperglycemic model

Core Tip: This study revealed that microRNA-630 (miR-630) expression in the renal tissue was significantly lower in diabetic kidney disease (DKD) rats than in normal rats. The miR-630 alleviates renal injury and inflammatory reactions in DKD rats by targeting toll-like receptor 4. Our findings provide new insights into the pathogenesis of DKD indicating that miR-630 may be a potential noninvasive biomarker for diagnosing and predicting the prognosis of DKD.

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INTRODUCTION

Diabetic kidney disease (DKD), a chronic kidney condition due to diabetes, is characterized by a steady decline in glomerular filtration rate and the onset and progression of albuminuria. End-stage renal failure is more common in patients with DKD than with chronic glomerulonephritis[1-4]. The primary early morphological alterations in DKD include glomerular and tubular hypertrophy, thickening of the glomerular basement membrane, fusion of foot processes, and growth of the mesangial matrix. These alterations progress to various degrees of tubulointerstitial fibrosis, which ultimately causes the loss of renal function[5]. However, the molecular mechanisms underlying DKD remain unclear.

Renal tubular epithelial cell (TEC) damage has been linked to DKD[6]. The TEC epithelial mesenchymal transition (EMT), a key mechanism in renal interstitial fibrosis, has gained recent attention[7]. EMT is caused by a complex interaction between several variables, including the inflammatory response, hypoxia, oxidative stress, growth factors, signaling pathways, microRNA (miRNA), and transcription factors Snail, Slug and Twist[8]. Therefore, focusing on these pathways may help comprehend the molecular mechanisms underlying damage to TECs in DKD.

The miRNAs are small noncoding RNAs consisting of 19-23 nucleotides that negatively regulate posttranscriptional gene expression by targeting the 3′-untranslated regions (3′UTR) of protein-coding messenger RNA (mRNA) transcripts; which play important roles in different physiological and pathological processes[9,10]. Because miRNA imbalance is directly associated with pathological processes in DKD, miRNAs may serve as diagnostic biomarkers and therapeutic targets. For instance, DKD is promoted by increased expression of toll-like receptor 4 (TLR4) when miR-203 is expressed at low levels[11]. The overexpression of miR-92b can minimize renal fibrosis and restore miR-92b expression to normal levels in the kidneys of mice with DKD[12].

The miR-630, a recently identified miRNA, is closely linked to tumor cell development and apoptosis and demonstrates aberrant expression in various malignancies, including liver cancer, colon cancer, gastric cancer, and other tumors[13,14]. Liu *et al*[15] reported that miR-630 targets TLR4 in immunoglobulin A (IgA) nephropathy to control the production of glycosylated IgA1 in tonsils. Studies on the expression and function of miR-630 in DKD-related renal tissue and the underlying pathophysiological mechanisms are lacking.

In this study, we assessed the relative expression of miR-630 mRNA in the kidney tissue of DKD rats and found that its expression was considerably lower than that in normal rats. Mechanistic studies have indicated TLR4 as the target gene for miR-630; therefore, miR-630 can be considered a possible pharmacological target for DKD treatment and a non-invasive biomarker for diagnosing DKD and determining its prognosis.

MATERIALS AND METHODS

Animals and cells

Sixty specific pathogen-free male Sprague-Dawley rats (6-wk-old and weighing 200 ± 20 g), were provided by the Experimental Animal Center of Jiangsu University. The rats were housed in the experimental animal feeding room at a temperature of 21 °C, humidity of 55 °C, and a 12-h light/dark period. Prior to the experiments, all rats were fed the same diet for one week. The rat renal TECs (NRK-52E) cell line was purchased from the Treasure Cell Bank of the China Academy of Sciences. The rat renal TECs (NRK-52E) cell line was purchased from the TreasureCell Bank of the China Academy of Sciences.

Reagents

Streptozotocin (STZ) was purchased from Shanghai Aiyan Biotechnology Co., Ltd. The citrate buffer was purchased from Beijing Noble Food Technology Co., Ltd. Lentiviral negative control (LV-NC), LV-miR630 and primers were all purchased from ABclonal. RIPA lysis buffer and BCA kits were purchased from Beyotime. The TLR4 antibody was purchased from Affinity. Enzyme-linked immunosorbent assay (ELISA) kits for interleukin-6 (IL-6), IL-1β and tumor necrosis factor-α (TNF-α) were purchased from Mlbio.

Instruments

The BK-200 automatic biochemical analyser was purchased from BIOBASE, the DR3518G enzyme-labelled instrument was purchased from Wuxi HiwellDiatek, the FluorChem HD2 gel imaging system was purchased from protein simple, and the CytoFLEXS flow cytometry was purchased from Beckman.

Methods

Model construction and processing: The experimental rats were randomly divided into a control group, model (DKD), model + negative control (NC) agomir (LV-NC), and model + miR-630 agomir (LV-miR-630) groups (n = 15 in each group). Six-week-old rats were fed adaptively for one week. Following a 12-h fast, the rats in the model and experimental groups received intraperitoneal injections of 60 mg/kg STZ solution to establish the DKD model, whereas the control group was injected with the same volume of sodium citrate buffer (0.1 mmol/L)[16]. After 72 h, blood was collected from the tail vein for analyzing glucose levels, and serum glucose ≥ 16.7 mmol/L was used for establishing the diabetic model. Urine was collected for 24 h, and the urine protein content was > 30 mg/24 h, for successful DKD modelling[17]. The rats in each experimental group were administered 100 μL of agomir NC and miR-630 agomir (50 nM) intravenously at weeks 2 and 5 after STZ injection, whereas rats in the model group received intravenous injections of equal volumes of normal saline. After 8 wk, the rats in each group were fasted for 12 h before blood samples were collected from the tail vein to detect fasting blood glucose. Urine was collected in a metabolic cage for 24 h 1 d before execution. The rats were injected intraperitoneally with 3% pentobarbital sodium (30 mg/kg), blood was collected from the abdominal aorta and kidneys, and the rats were sacrificed. The renal tissue was collected for further analysis.

Indicator monitoring: The mental state, color change of the hair and nails, activity, urine volume, and weight of rats were observed during administration. On the last day of the experiment, 24-h urine samples of the rats in a metabolic cage. After mixing, the samples were centrifuged at 3000 rpm (centrifugal radius: 16.5 cm) for 10 min, and the supernatant was collected to detect the 24-h urine protein quantification. Random blood glucose levels were measured, and the rats were anesthetized using an intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg). Blood was collected from the abdominal aorta and centrifuged at 4 °C and 3500 rpm (centrifugal radius: 16.5 cm) for 15 min. Serum blood urea nitrogen (BUN) and creatinine levels were measured using a kit.

Calculation of renal weight index: The kidneys were washed with precooled normal saline, dried using filter paper and weighed, and the rat kidney index was calculated using the formula: Kidney index (%) = (total weight of bilateral

kidneys/weight of rats) × 100.

Quantitative reverse transcription polymerase chain reaction detection of miR-630 and TLR4 mRNA expression

The TRIzol method was used to extract total RNA from each group. The expression of miR-630 and TLR4 mRNA was detected using a one-step reverse transcription fluorescence quantitative kit (Table 1 for primers). The reaction system and quantitative reverse transcription polymerase chain reaction (qRT-PCR) procedures were performed according to the manufacturer's instructions, and $2^{-\Delta\Delta Ct}$ method was used for relative quantitative analysis, with U6 and GAPDH as internal references.

Luciferase reporter assay

For the construction of wild-type and mutant TLR4 3′-UTR double-fluorescent reporter plasmids, 293T cells in the logarithmic growth period were inoculated in a 12-well cell plate at a density of 1×10^5 cells/well. Negative controls of TLR4-WT, TLR4-MUT, and miR-630 mimics or mimic NC were transfected into 293T cells according to the manufacturer's instructions for LipofectamineTM2000. Three replicates were performed for each group of experiments. After 48 h of incubation, luciferase activity was detected using a double luciferase reporter gene detection kit.

Western blotting to detect TLR4 expression in renal tissue

RIPA lysate was added to the tissues of each group, placed on ice for 20 min, and centrifuged at 4 °C and 13000 rpm at 4 °C for 20 min, and the protein content in the supernatant was determined using a BCA kit. The sodium-dodecyl sulfate gel electrophoresis was performed with a 35-μg protein solution, transferred to the polyvinylidene fluoride membrane, and sealed with a 2% BSA sealing solution. The primary antibodies were added at 4 °C and left overnight. The GAPDH antibody was used as the reference, and secondary antibodies were added and incubated for 1 h at room temperature. Enhanced chemiluminescence exposure imaging was performed using an Alpha Imager HP gel imaging system to analyze the results.

Hematoxylin and eosin staining to detect renal injury

The kidneys were fixed with 4% paraformaldehyde, routinely dehydrated, and embedded in paraffin. After dewaxing, the tissues were stained with hematoxylin and eosin (HE), followed by 1% hydrochloric acid ethanol differentiation, 0.6% ammonia return to blue, 0.5% eosin staining, conventional dehydration, xylene transparency, and neutral gum sealing. Renal injury was observed under a microscope (400 × magnification).

Masson staining to observe the histological changes in the kidney

The slices were dewaxed in water. Sections were stained with the prepared Weigert hematoxylin staining solution for 5-10 min, differentiated with an acidic ethanol differentiation solution for 5-15 s, and washed with water. The Masson blue-stained solution returned to blue after 3-5 min and was washed with water. After washing with distilled water for l min, the sections were stained with Ponceau magenta dye solution for 5-10 min. The weak acid working solution used in this procedure was prepared using a 2:1 ratio of distilled water to the weak acid solution. The sample was washed with the weak acid working solution for 1 min, then washed with the phosphomolybdic acid solution for 1-2 min, and washed again with the prepared weak acid working solution for l min. The sample was placed directly in the aniline blue dye solution for 1-2 min and then washed with the prepared weak-acid working solution for 1 min. The sample was quickly dehydrated using 95% ethanol for 2-3 s and anhydrous ethanol three times for 5-10 s each time. The sections were transparentized with xylene three times for 1-2 min each and sealed with neutral gum.

IL-6, IL-1β and TNF-α levels in renal tissue detected using ELISA

Renal tissue was ground on ice and transformed into a 10% tissue homogenate. The levels of IL-6, IL-1β and TNF-α in renal tissue were detected according to the instructions of the TNF-α ELISA kit.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 (IBM Corp., Armonk, NY, United States) and GraphPad Prism 9.0 software, and the results are expressed as the mean ± standard error (mean ± SEM) or mean ± SD. The *T*-test, one-way ANOVA, and SNK-*q* tests were used for intergroup and intragroup mean comparisons. A *P* value of 0.05 indicated statistical significance. Correlation analyses were performed using Pearson correlation analysis and linear regression.

RESULTS

General observation

Rats in the normal group were in a good mental state, lively and active, with bright eyes, sensitive reactions, and white and shiny fur. The model rats were depressed; their fur was yellow, dry, and dirty; their movements were slow; and they exhibited symptoms such as excessive drinking, excessive eating, excessive urination, and thin feces. Over time, some rats with DKD exhibited varying degrees of abdominal distension. Rats in the miR-630 agomir group had better general conditions than those in the model group.

Table 1 Primers for quantitative reverse transcription polymerase chain reaction

Primers	Sequence (5'-3')
TLR4 forward	TAGCCATTGCTGCCAACATC
TLR4 reverse	ACACCAACGGCTCTGGATAA
miR-630 forward	TTGAGCTGGATTGGCGGGAT
miR-630 reverse	TTGACGGATGCGGAGGCT
GAPDH forward	TATGTCGTGGAGTCTACTGTGT
GAPDH reverse	GAGTTGTCATATTTCTCGTGG
U6 forward	CATCACCATCAGGAGAGTCG
U6 reverse	TGACGCTTGCCCACAGCCTT

TLR4: Toll-like receptor 4.

Expression of miR-630 mRNA in kidney tissue of DKD rats and the pathological changes in kidney tissue

As demonstrated in Figure 1A, compared with the control group, miR-630 mRNA expression in the DKD group declined considerably, as did body weight, whereas blood glucose levels and the kidney weight index increased significantly (all $P < 0.01$). Pearson correlation analysis indicated that the level of miR-630 in rats was positively correlated with body weight and albumin but negatively correlated with renal weight index, urea nitrogen, serum creatinine (SCr), 24-h urine protein quantification, blood glucose, and other variables (Figure 1B). These findings imply that miR-630 expression in renal tissues is associated with clinical variables and may be associated with DKD. The results of the HE staining are shown in Figure 1C. The renal tissue cell structure remained unaltered in the control group, and no overt pathological alterations were observed. Enlarged or detached TECs, renal interstitial cell infiltration, mesangial hyperplasia, interstitial fibrosis, and glomerular edema were detected in the DKD group.

Overexpression of miR-630 inhibits TEC damage induced by high glucose in vitro

Figure 2A shows the expression levels of miR-630 and TLR4 in NRK-52E determined using qRT-PCR. The TLR4 and miR-630 mRNA expression levels in the normal glucose (NG) and high mannitol (HM) groups were not significantly different. Although miR-630 and TLR4 mRNA expression levels were significantly decreased in the high glucose (HG) and HG + mimic NC groups compared with those in the NG group, TLR4 mRNA expression levels considerably increased. Compared with the HG + miR-630 mimic NC group, the mRNA expression levels of miR-630 and TLR4 in the NG group substantially increased and decreased, respectively.

ELISA was used to determine the levels of TNF-α, IL-1β, and IL-6 in each group. The results are shown in Figure 2B. TNF-α, IL-1β, and IL-6 levels in the NG and HM groups were not altered considerably; however, the HG and HG + mimic NC groups demonstrated a significant increase than that observed in the NG group. The levels of TNF-α, IL-1β, and IL-6 in the HG + miR-630 mimic group were considerably lower than those in the HG + mimic NC group.

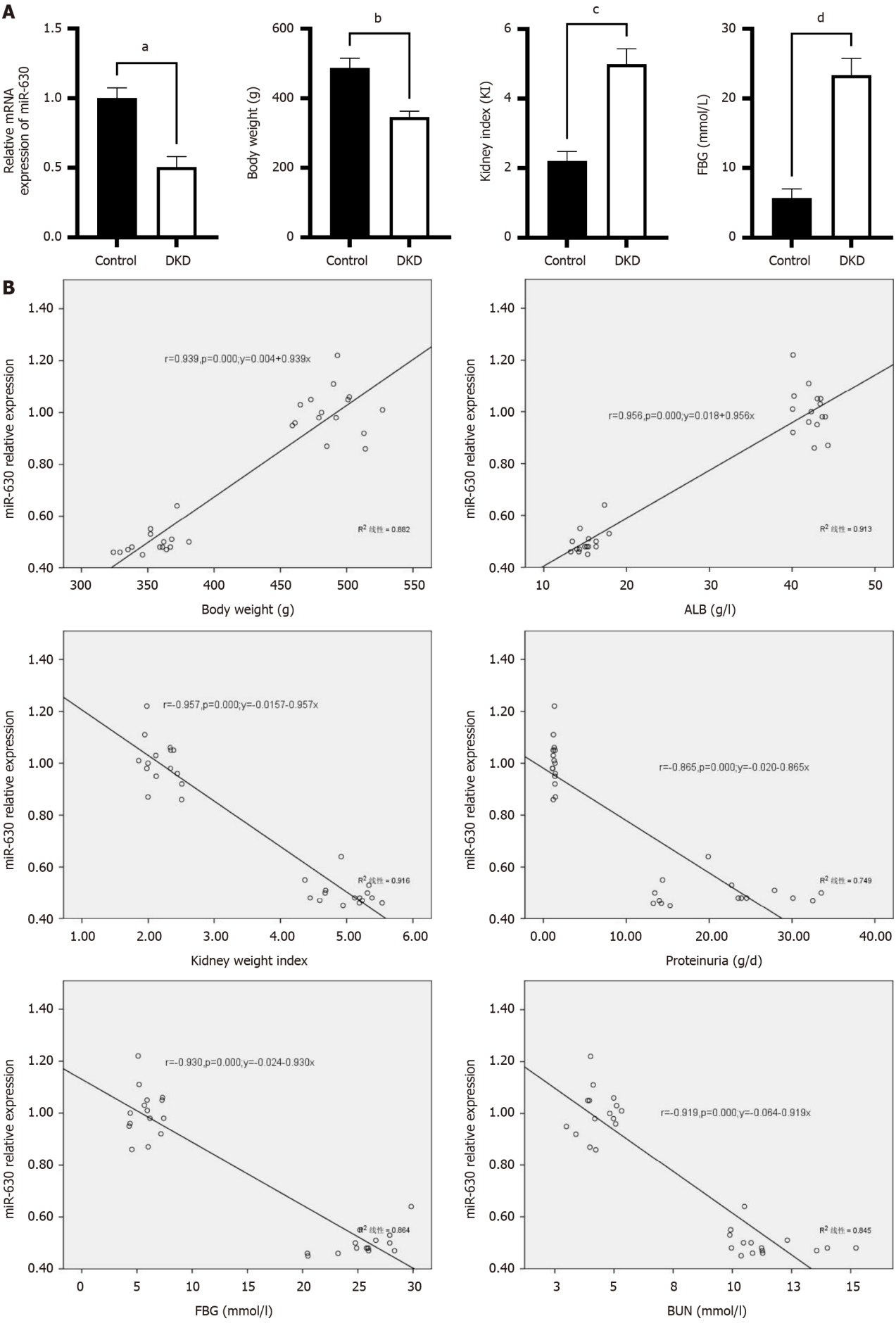
Figure 2C displays the results of western blot analysis used to determine the protein expression levels of TLR4, E-cadherin, E-smooth muscle actin (SMA), and collagen IV in each group. The expression levels of TLR4, E-cadherin, α-SMA, and collagen IV were not significantly altered in the NG and HM groups. E-cadherin expression levels in the HG group and HG + mimic NC group were significantly lower than those in the NG group, although TLR4, α-SMA, and collagen IV expression levels were significantly increased. Compared with the HG + mimic NC group, the expression levels of E-cadherin protein in the HG + miR-630 mimic group increased substantially, whereas those of TLR4, α-SMA, and collagen IV protein decreased significantly.

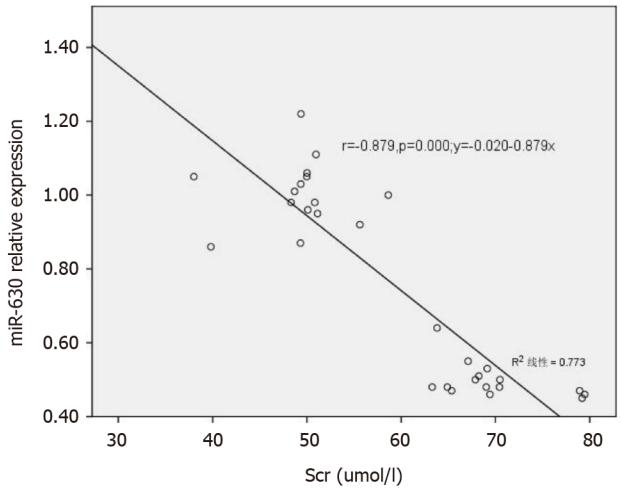
miR-630 targeted the downregulation of TLR4

TargetScan and other databases predicted that miR-630 has a binding site in the 3'-UTR of TLR4 (Supplementary Table 1 and Supplementary Figure 1) (Figure 3A). Based on the experimental findings using a double luciferase reporter gene, high levels of miR-630 substantially reduced the luciferase activity of the wild-type TLR4 plasmid ($P < 0.01$) but had no effect on the mutant TLR4 plasmid (Figure 3B).

Figure 3C shows the levels of miR-630 and TLR4 mRNA expression determined using qRT-PCR. Compared with the HG + mimic NC group, the mRNA expression of miR-630 increased significantly in the HG + miR-630 mimic group, whereas the mRNA expression of TLR4 was significantly decreased. The mRNA expression of miR-630 in the HG + miR-630 mimic + oe-TLR4 group was considerably lower than that in the HG + miR-630 mimic + oe-NC group; however, the mRNA expression of TLR4 was dramatically higher.

The ELISA results showed that miR-630 downregulated the levels of INF-α, IL-1 β, and IL-6 in TLR4 (Figure 3D). The levels of INF-α,IL-1 β, and IL-6 in the HG + miR-630 mimic group were considerably lower than those in the HG + mimic NC group. Compared with the HG + miR-630 mimic + oe-NC group, the TNF-α, IL-1β, and IL-6 levels were significantly increased in the HG + miR-630 mimic + oe-TLR4 group.


A
Relative mRNA expression of miR-630
a
Control
DKD
Body weight (g)
b
Kidney index (KI)
c
FBG (mmol/L)
d
B
miR-630 relative expression
r=0.939,p=0.000;y=0.004+0.939x
Body weight (g)
r=0.956,p=0.000;y=0.018+0.956x
ALB (g/l)
r=-0.957,p=0.000;y=-0.0157-0.957x
Kidney weight index
r=-0.865,p=0.000;y=-0.020-0.865x
Proteinuria (g/d)
r=-0.930,p=0.000;y=-0.024-0.930x
FBG (mmol/l)
r=-0.919,p=0.000;y=-0.064-0.919x
BUN (mmol/l)



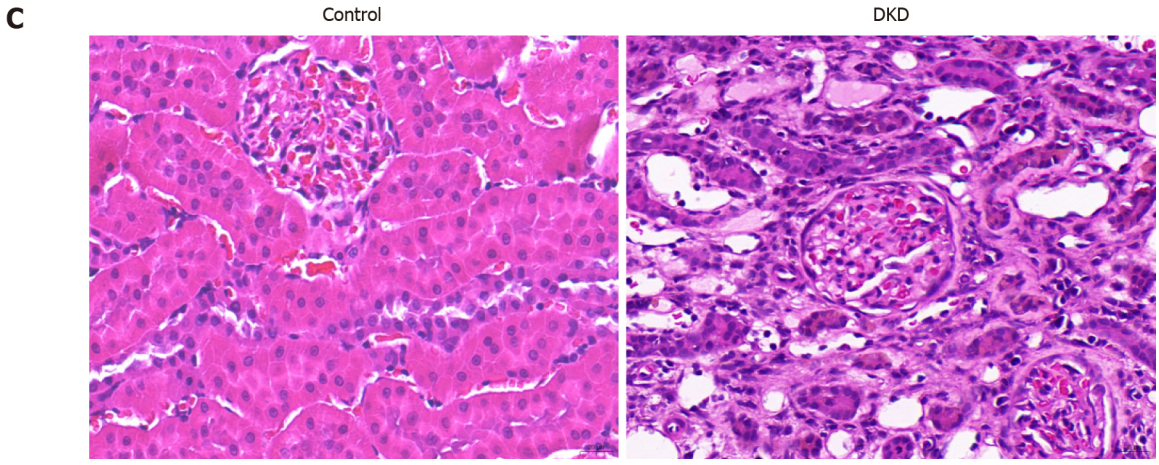


Figure 1 The expression of miR-630 in diabetic kidney disease rats. A: Differential expression of miR-630, renal weight index, and blood glucose in diabetic kidney disease rats; B: The correlations between the expression of miR-630 and clinical parameters, including body weight, serum albumin, renal weight index, blood urea nitrogen, serum creatinine and proteinuria, were analysed by Pearson correlation analysis and linear regression analysis; C: Pathological changes in renal tissue in rats under a high glucose environment (hematoxylin and eosin, × 400). n = 15. Data are presented as mean ± SD, $^{a}P < 0.001$, $^{b}P < 0.001$, $^{c}P < 0.001$, $^{d}P < 0.001$. DKD: Diabetic kidney disease.

Western blotting was used to detect the protein expression levels of TLR4, α-SMA, collagen IV and E-cadherin, which were downregulated by miR-630 (Figure 3E). Compared with the HG + mimic NC group, the protein expression levels of TLR4, α-SMA and collagen IV in the HG + miR-630 mimic group decreased significantly, whereas the protein expression level of E-cadherin increased significantly. Compared with the HG + miR-630 mimic + oe-NC group, the expression levels of TLR4, α-SMA and collagen IV proteins in the HG + miR-630 mimic + oe-TLR4 group were significantly increased, whereas the expression level of E-cadherin protein was significantly decreased.

Overexpression of miR-630 improves the biochemical changes in DKD model rats

Figure 4A summarizes the body weight, renal weight index, blood sugar, 24-h urinary protein, BUN, and SCr. No significant differences in body weight, renal weight index, blood glucose, 24-h urinary protein, BUN, or SCr levels was observed between the DKD and DKD + NC agomir groups. Compared with the DKD + NC agomir group, the body weight increased significantly in the DKD + miR-630 agomir group, and the renal index decreased significantly. As shown in Figure 4A, no significant differences in blood glucose levels were observed between the DKD and DKD + NC agomir groups. Compared with the DKD + NC agomir group, blood glucose levels in the DKD + miR-630 agomir group decreased significantly. Figure 4C summarizes the results of the automatic biochemical analyzer; no significant difference in 24-h urine protein, BUN, and SCr levels were observed between the DKD and DKD + NC agomir groups. Compared with the DKD + NC agomir group, the contents of 24-h urine protein, BUN, and SCr levels in the DKD + miR-630 agomir group decreased significantly.

Effects of miR-630 overexpression on TLR4, TNF-α, IL-1β,and IL-6 in DKD rats

The mRNA expression levels of miR-630 and TLR4 were measured using qRT-PCR. As shown in Figure 4B, no significant differences in the mRNA expression levels of miR-630 and TLR4 were observed between the DKD and the DKD + NC agomir groups. Compared with the DKD + NC agomir group, the mRNA expression of miR-630 in the DKD + miR-630 agomir group increased significantly, whereas the mRNA expression of TLR4 decreased significantly. The protein levels

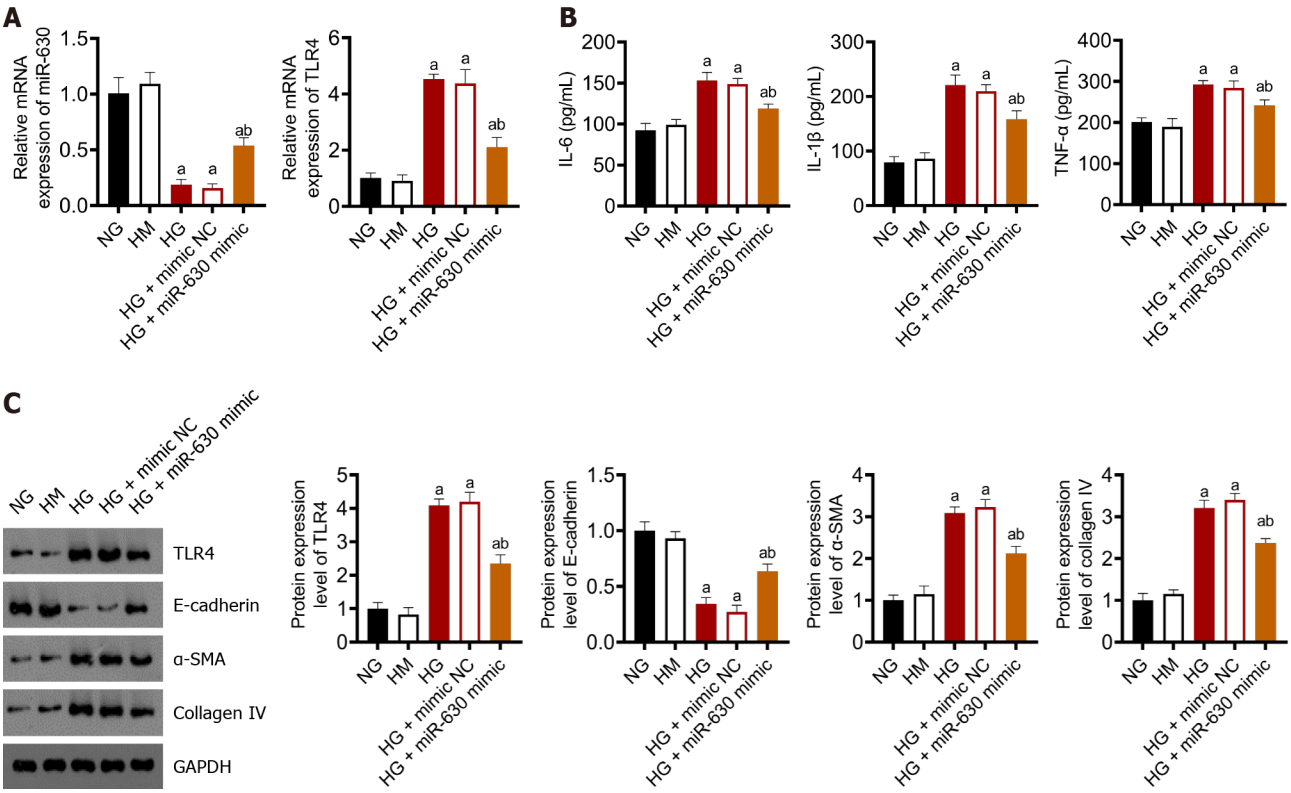


Figure 2 Overexpression of miR-630 inhibits renal tubular epithelial cell damage induced by high glucose *in vitro*. A: Quantitative reverse transcription polymerase chain reaction detection of miR-630 and toll-like receptor 4 (TLR4) expression in rat renal tubular epithelial cells in a high glucose environment (NRK-52E); B: Detection of tumor necrosis factor-α, interleukin (IL)-1β and IL-6 expression in rat renal tubular epithelial cells (NRK-52e) under a high glucose environment by enzyme-linked immunosorbent assay; C: Western blot detection of NRK-52E TLR4 in rats under a high glucose environment α-smooth muscle actin and collagen IV protein expression. Data are presented as mean ± SD, $^{a}P < 0.01$ *vs* normal glucose, and $^{b}P < 0.01$ *vs* high glucose + mimic normal glucose. NG: Normal glucose, 5.6 mmol/L; HG: High glucose, 20 mmol/L; HM: High mannitol (5.6 mmol/L glucose + 14.4 mmol/L mannitol); TLR4: Toll-like receptor 4; α-SMA: α-smooth muscle actin; IL: Interleukin; TNF: Tumor necrosis factor.

of IL-6, IL-1β, and TNF-α were detected by ELISA. As shown in Figure 4C, no significant difference in the contents of IL-6, IL-1β, and TNF-α was observed between the DKD group and the DKD + NC agomir group. Compared with the DKD + NC agomir group, the contents of IL-6, IL-1β, and TNF-α in the DKD + miR-630 agomir group decreased significantly. The expression levels of TLR4, E-cadherin, α-SMA, and collagen IV were detected by western blotting. As shown in Figure 4D, no significant difference in the expression levels of TLR4, E-cadherin, α-SMA and collagen IV was observed between the DKD group and the DKD + NC agomir group. Compared with the DKD + NC agomir group, the expression of E-cadherin protein in the DKD + miR-630 agomir group was significantly increased, whereas the expression of TLR4, α -SMA, and collagen IV protein was significantly decreased.

Effects of overexpression of miR-630 on glomerular morphology in DKD rats

The results of the HE test are shown in Figure 5A. In the DKD and DKD + NC agomir groups, glomerular swelling, TEC swelling or falling off, renal interstitial cells infiltrating inflammatory cells, and some mesangial hyperplasia and interstitial fibrosis were observed. Renal pathological changes were alleviated and were accompanied by a small amount of inflammatory cell infiltration in the DKD + miR-630 agomir group. The Masson test results are shown in Figure 5B, and many blue-stained collagen fibers appeared in the renal glomeruli of rats in the DKD and DKD + NC agomir groups. Few blue-stained collagen fibers in the kidney tissue of rats were observed in the DKD + miR-630 agomir group.

DISCUSSION

The prevalence of DKD has been increasing globally, with significant morbidity and mortality. The pathophysiology of DKD is complex and has not been elucidated to date. Numerous studies have now established the role of miRNA in the occurrence and progression of diabetic nephropathy[18]. The expression levels of miR-21, miR-146a-5p, miR-10a-5p, miR-874, and miR-192 are significantly increased in diabetic nephropathy, whereas miR-26a-5p, miR-451, and miR-155 are expressed at low levels[17,19-21]. Among them, miR-21 functions by targeting the PTEN gene, thereby promoting the activation of the Akt kinase signaling pathway, which in turn increases the production of the renal fibrosis proteins type I collagen a2 and mucin and glomerular hypertrophy[17]. By targeting the ZEB1/2 gene, miR-192 activates the transforming growth factor-beta signaling pathway, increasing the transcription of the renal fibrosis protein Coll2 and the

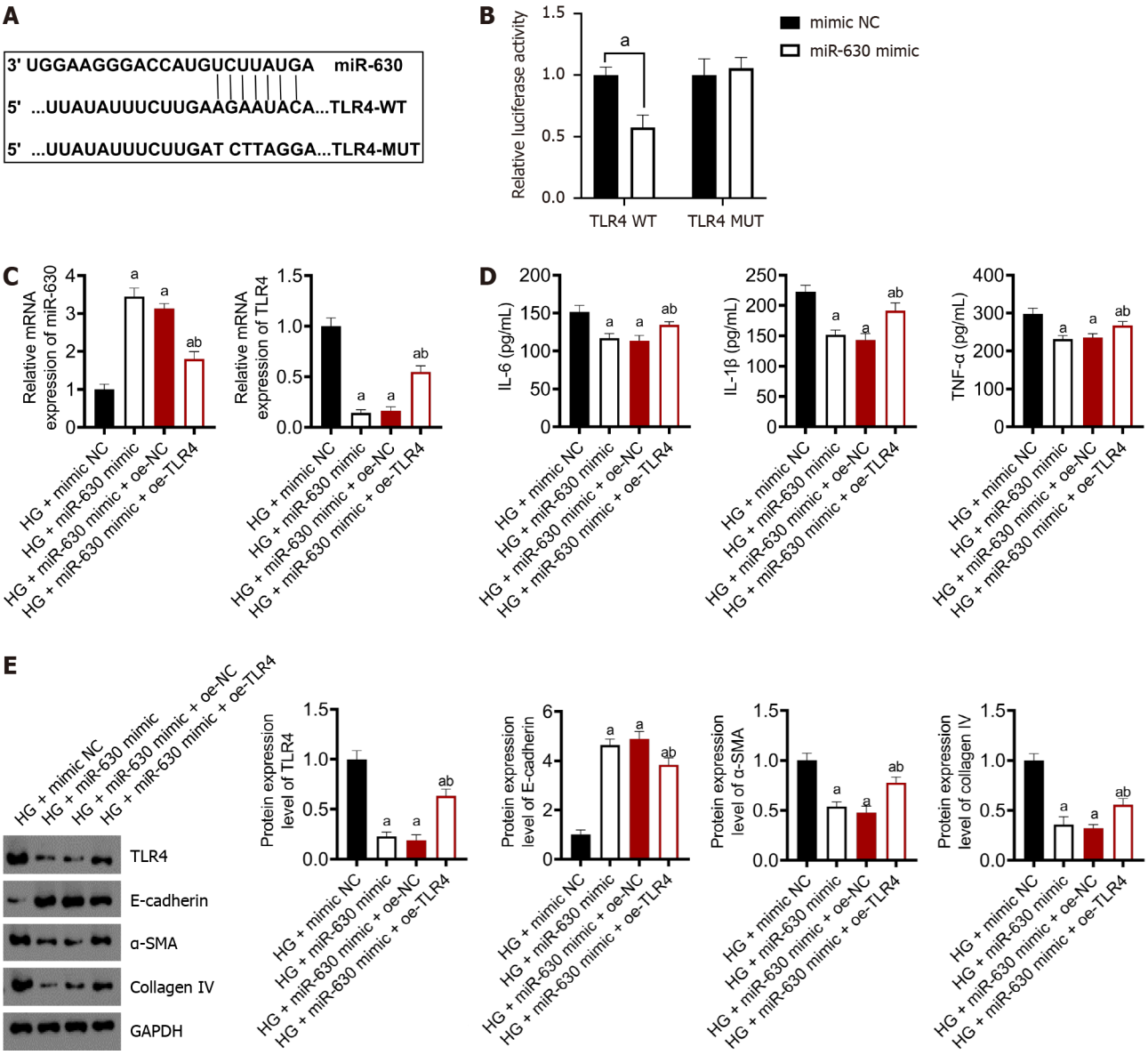


Figure 3 Toll-like receptor 4 is the target gene of miR-630. A: Bioinformatic analysis showed the putative miR-630 target sites in the toll-like receptor 4 (TLR4) 3'-untranslated regions (UTR). The mutated nucleotides are underlined; B: The WT-TLR4 3'-UTR and the MUT-TLR4 3'-UTR reporters were cotransfected with miR-630 mimic or negative control into NRK-52Es. Forty-eight hours after transfection, the luciferase activities were measured; C: Quantitative reverse transcription polymerase chain reaction detection of miR-630-targeted downregulation of TLR4 mRNA expression levels; D: Enzyme-linked immunosorbent assay detection of tumor necrosis factor in miR-630-targeted downregulation of TLR4, α-smooth muscle actin (SMA), interleukin (IL)-1β, and IL-6 content; E: Western blotting was used to detect the protein expression of TLR4, α-SMA, collagen IV and E-cadherin downregulated by miR-630. Data are presented as mean ± SD, $^{a}P < 0.01$ *vs* high glucose + mimic normal glucose, $^{b}P < 0.01$ *vs* high glucose + miR-630 mimic + oe normal glucose. NG: Normal glucose, 5.6 mmol/L; HG: High glucose, 20 mmol/L; HM: High mannitol (5.6 mmol/L glucose + 14.4 mmol/L mannitol); TLR4: Toll-like receptor 4; α-SMA: α-smooth muscle actin; IL: Interleukin; TNF: Tumor necrosis factor; NC: Negative control.

amount of albumin in urine[21].

The recently identified miRNA, miR-630, is a noncoding single-stranded RNA fragment with a length of 21-23 nucleotides that regulates gene expression at the translational level and participates in several pathophysiological processes, including cell proliferation and differentiation[22], apoptosis[23], and immune response[24]. Aberrant expression of the miR-630 gene has been reported in numerous malignancies, such as liver, colon, and gastric cancers[14,25]. Moreover, miR-630 is strongly associated with autophagy, cell proliferation, migration, and apoptosis[26-28]. However, the expression and functions of miR-630 in the renal tissues of patients with DKD are unknown. A rat model of DKD was created by intraperitoneally injecting STZ. The 24-h urinary total protein, SCr, and BUN levels increased and the model rats manifested clear signs of diabetes. The model was successful because it revealed the characteristic renal pathological abnormalities of DKD when stained with Masson's trichrome and HE. Furthermore, the expression of miR-630 was investigated, which was much lower in DKD renal tissue than in normal renal tissue, indicating that miR-630 may play a role in the pathogenesis of DKD.

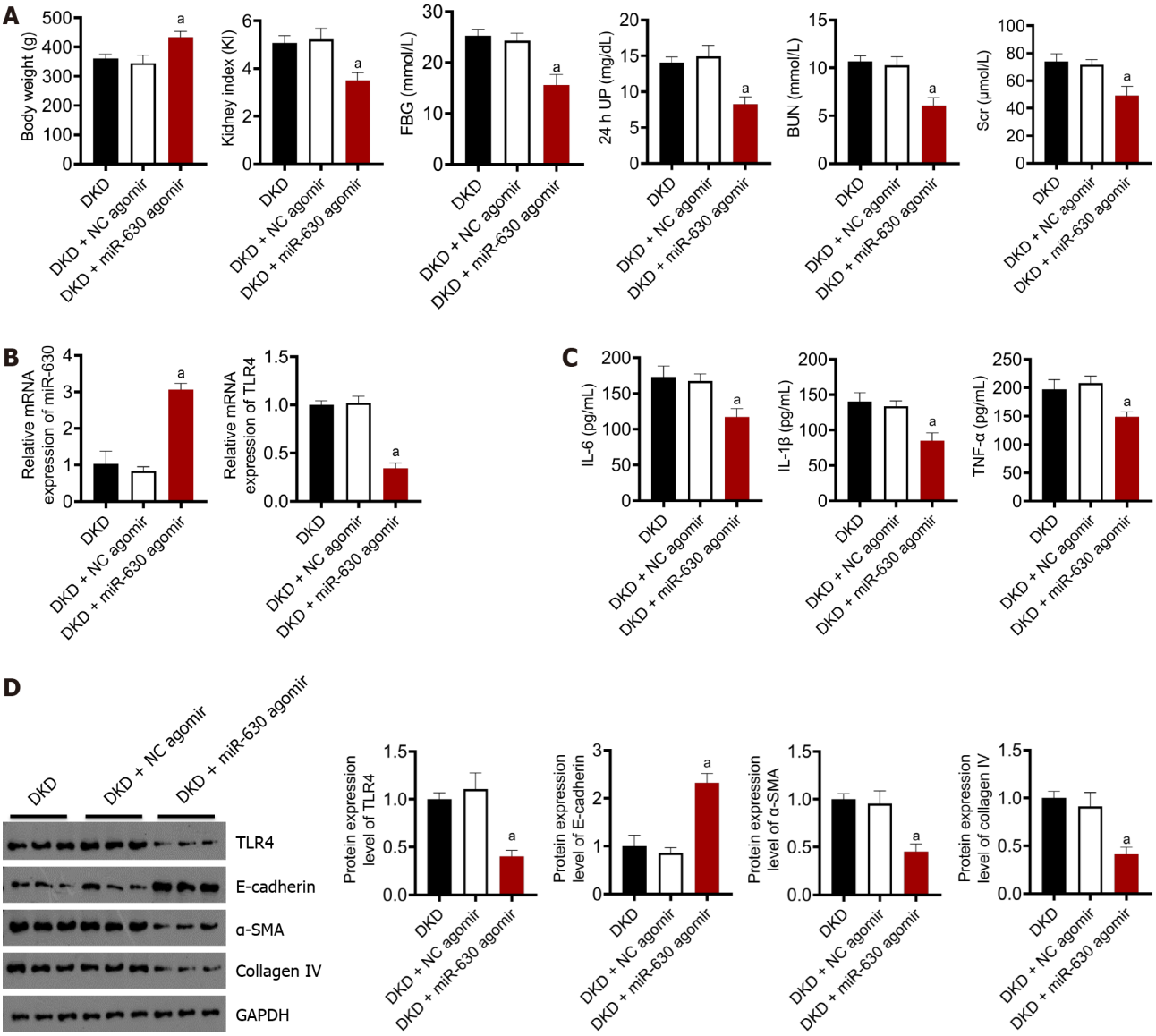


Figure 4 Overexpression of miR-630 improves the biochemical changes in diabetic kidney disease model rats. A: Effects of miR-630 overexpression on body weight, renal weight index, blood glucose, fasting blood glucose, 24-h urinary protein, blood urea nitrogen and serum creatinine in rats; B: Effect of overexpression of miR-630 on the expression of toll-like receptor 4 (TLR4) mRNA in diabetic kidney disease rats; C: MiR-630 was overexpressed, and the levels of interleukin (IL)-6, IL-1β and tumor necrosis factor-α were detected by enzyme-linked immunosorbent assay; D: MiR-630 was overexpressed, and the expression levels of TLR4, E-cadherin, α-smooth muscle actin and collagen IV were detected by western blotting. Data are presented as mean ± SD, $^{a}P < 0.01$ *vs* diabetic kidney disease + normal glucose agomir. DKD: Diabetic kidney disease; FBG: Fasting blood glucose; 24 h UP: 24-h urinary protein; BUN: Blood urea nitrogen; Scr: Serum creatinine; TLR4: Toll-like receptor 4; α-SMA: α-smooth muscle actin; IL: Interleukin; TNF: Tumor necrosis factor; NC agomir: Negative control of lentivirus.

Inflammation is crucial for the pathogenesis of DKD[29]. TLR4, the first confirmed member of the toll-like family in humans, can activate signaling pathways such as the nuclear factor-kappa B and mitogen-activated protein kinase family pathways after interacting with ligands *in vivo*, which increases the production of inflammatory markers and activates an inflammatory response[30]. Activation of the TLR4 signaling pathway is closely related to the pathogenesis of diabetic nephropathy, and the expression of TLR4 and related inflammatory factors TNF-α, IL-6, and IL-1β increases during the occurrence and development of diabetic nephropathy[31,32]. This work used a bioinformatics website to predict that miR-630 may combine with the 3′-UTR of TLR4 and a twofold luciferase assay to confirm that TLR4 was the direct target of miR-630. Overexpression of miR-630 in DKD rats caused a decrease in TLR4 expression and the levels of the proinflammatory molecules TNF-α, IL-6, and IL-1β, demonstrating a negative regulatory link between miR-630 and TLR4. To prevent TLR4 from being translated and transcribed, miR-630 attaches to its 3′-UTR on the mRNA, which inhibits the synthesis of the proinflammatory protein TNF-α. In addition, overexpression of miR-630 in DKD rats led to an improvement in general health, an increase in weight, a drop in renal index, an improvement in urine protein and renal function, and a reduction in renal pathological damage. Consequently, miR-630 overexpression could reduce the inflammatory response and mesenchymal trans-differentiation of diabetic nephropathy. Moreover, the contents of TNF-α, IL-6, and IL-1β significantly decreased, and the expression of the renal tubular epithelial marker protein E-cadherin increased, whereas the expression of the mesenchymal marker proteins α-SMA and collagen IV decreased.

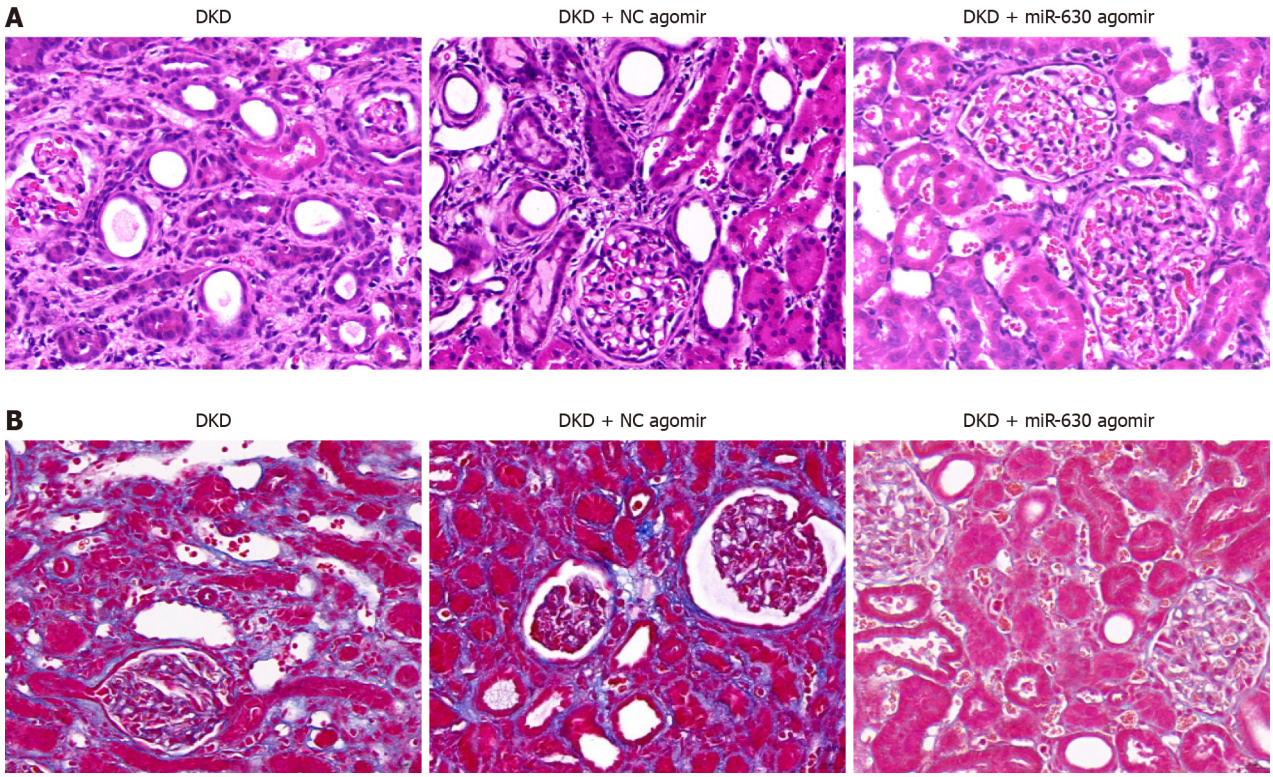


Figure 5 Effects of overexpression of miR-630 on glomerular morphology in diabetic kidney disease rats. A: Effect of overexpression of miR-630 on glomerular morphology in diabetic kidney disease (DKD) rats (hematoxylin and eosin, × 200); B: Effect of overexpression of miR-630 on glomerular morphology in DKD rats (Masson, × 200). DKD: Diabetic kidney disease; NC agomir: Negative control of lentivirus.

In addition, as shown in Figure 4A, overexpression of miR-630 can significantly reduce blood glucose levels, which may prevent the progression of DKD. However, the role of miR-630 in promoting insulin secretion has not been demonstrated, which requires further study and exploration. In conclusion, this study showed that miR-630 targets TLR4, and inhibits the inflammatory response that results in DKD, and exerts protective effects on the kidney under diabetic conditions.

CONCLUSION

To our knowledge, our study was the first to report that the expression of miR-630 in renal tissue is significantly lower in DKD rats than in normal rats. These results revealed the underlying mechanism by which miR-630 alleviates renal injury and inflammatory reactions in rats with DKD by targeting TLR4. Taken together, our findings provide new insights into the pathogenesis of DKD and show that miR-630 may be a non-invasive biomarker for the diagnosis and prediction of the prognosis of DKD.

ARTICLE HIGHLIGHTS

Research background

Diabetic kidney disease (DKD) is a major complication of diabetes mellitus. Numerous studies have demonstrated that tubular epithelial cell (TEC) damage, which is strongly associated with the inflammatory response and mesenchymal trans-differentiation, plays a significant role in DKD; however, the precise molecular mechanism is unknown. The recently identified microRNA-630 (miR-630) has been hypothesized to be closely associated with cell migration, apoptosis, and autophagy.

Research motivation

The relationship between miR-630 and DKD and the underlying mechanism remains unknown.

Research objectives

The object of this study is to investigate how miR-630 affects TEC injury and the inflammatory response in DKD rats.

Research methods

Streptozotocin was administered to six-week-old male rats to create a hyperglycemic diabetic model, and in the second week of modeling, the rats were divided into control, DKD, negative control lentivirus, and miR-630 overexpression groups. After eight weeks, urine and blood samples were collected for the kidney injury assay, and renal tissues were removed for further molecular assays, such as real-time polymerase chain reaction, western blotting, enzyme-linked immunosorbent assay, and immunohistochemistry. The target gene for miR-630 was predicted using bioinformatics, and *in vitro* investigations and double luciferase reporter gene assays confirmed the association between miR-630 and toll-like receptor 4 (TLR4).

Research results

The expression level of miR-630 was decreased in the kidney tissue of rats with DKD ($P < 0.05$). *In vitro* experiments, the mRNA expression level of miR-630 was significantly lower in the high glucose (HG) and HG + mimic negative control (NC) groups than in the normal glucose group ($P < 0.05$). In contrast, the mRNA expression level of TLR4 was significantly higher in these groups ($P < 0.05$). The HG and HG + mimic NC groups showed a significant decrease in E-cadherin protein expression, whereas TLR4, α-smooth muscle actin (SMA), and collagen IV protein expression increased ($P < 0.05$). Conversely, compared with the HG + mimic NC group, a significant increase in E-cadherin protein expression and a notable decrease in TLR4, α-SMA, and collagen IV protein expression were observed in the HG + miR-630 mimic group ($P < 0.05$). *In vivo* experiments, DKD rats treated with miR-630 agomir exhibited significantly higher miR-630 mRNA expression than DKD rats injected with agomir NC. Additionally, rats treated with miR-630 agomir showed significant reductions in urinary albumin, blood glucose, TLR4, and proinflammatory markers (TNF-α, IL-1β, and IL-6) expression levels ($P < 0.05$). Moreover, these rats exhibited fewer kidney lesions and reduced infiltration of inflammatory cells.

Research conclusions

The miR-630 may inhibit the inflammatory reaction in DKD by targeting TLR4, and has a protective effect on DKD.

Research perspectives

The follow-up study needs to further confirm the expression difference of clinical human blood, urine or kidney tissue in diabetic nephropathy patients and its relationship with DKD staging, and further clarify the regulatory mechanism of its upstream signal pathway.

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FOOTNOTES

Author contributions: Wu QS conceived and designed the experiments. Wu QS and Zheng DN performed the experiments, analysed the data, and prepared all the figures; Ji C, Qian H, Jin J, and He Q provided technical support; Wu QS wrote the manuscript; and all authors contributed to the article and approved the submitted version.

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ORIGINAL ARTICLE

Basic Study

Jianpi Gushen Huayu decoction ameliorated diabetic nephropathy through modulating metabolites in kidney, and inhibiting TLR4/NF-κB/NLRP3 and JNK/P38 pathways

Zi-Ang Ma, Li-Xin Wang, Hui Zhang, Han-Zhou Li, Li Dong, Qing-Hai Wang, Yuan-Song Wang, Bao-Chao Pan, Shu-Fang Zhang, Huan-Tian Cui, Shu-Quan Lv

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Abstract

BACKGROUND

Jianpi Gushen Huayu Decoction (JPGS) has been used to clinically treat diabetic nephropathy (DN) for many years. However, the protective mechanism of JPGS in treating DN remains unclear.

AIM

To evaluate the therapeutic effects and the possible mechanism of JPGS on DN.

METHODS

We first evaluated the therapeutic potential of JPGS on a DN mouse model. We then investigated the effect of JPGS on the renal metabolite levels of DN mice using non-targeted metabolomics. Furthermore, we examined the effects of JPGS on c-Jun N-terminal kinase (JNK)/P38-mediated apoptosis and the inflammatory

responses mediated by toll-like receptor 4 (TLR4)/nuclear factor-kappa B (NF-κB)/NOD-like receptor family pyrin domain containing 3 (NLRP3).

RESULTS

The ameliorative effects of JPGS on DN mice included the alleviation of renal injury and the control of inflammation and oxidative stress. Untargeted metabolomic analysis revealed that JPGS altered the metabolites of the kidneys in DN mice. A total of 51 differential metabolites were screened. Pathway analysis results indicated that nine pathways significantly changed between the control and model groups, while six pathways significantly altered between the model and JPGS groups. Pathways related to cysteine and methionine metabolism; alanine, tryptophan metabolism; aspartate and glutamate metabolism; and riboflavin metabolism were identified as the key pathways through which JPGS affects DN. Further experimental validation showed that JPGS treatment reduced the expression of TLR4/NF-κB/NLRP3 pathways and JNK/P38 pathway-mediated apoptosis related factors.

CONCLUSION

JPGS could markedly treat mice with streptozotocin (STZ)-induced DN, which is possibly related to the regulation of several metabolic pathways found in kidneys. Furthermore, JPGS could improve kidney inflammatory responses and ameliorate kidney injuries in DN mice *via* the TLR4/NF-κB/NLRP3 pathway and inhibit JNK/P38 pathway-mediated apoptosis in DN mice.

Key Words: Diabetic nephropathy; Jianpi Gushen Huayu Decoction; Oxidative stress; Inflammation; Untargeted metabolomics; Toll-like receptor 4/nuclear factor-kappa B/NOD-like receptor family pyrin domain containing 3 pathway; c-Jun N-terminal kinase/P38-mediated apoptosis

Core Tip: Traditional Chinese medicine (TCM) has been demonstrated to possess beneficial effects on diabetes and its complications. Elucidating upon these mechanisms can contribute to the modernization of TCM. The dysfunction of metabolism is closely related to the progression of diabetes and diabetic complications. Using untargeted metabolomics can be useful in studying the metabolic regulatory mechanisms of TCM. The current study used untargeted metabolomics to evaluate the differential metabolites in a diabetic nephropathy (DN) mouse model after Jianpi Gushen Huayu Decoction (JPGS) treatment. Moreover, we deeply analyzed the results from metabolomics and tested the potential pathways related to the differential metabolites. Our results revealed that JPGS could markedly treat mice with streptozotocin (STZ)-induced DN. The metabolomics results exhibited that the efficacy of JPGS is possibly related to cysteine and methionine metabolism; alanine, aspartate, and glutamate metabolism; tryptophan metabolism; and riboflavin metabolism.

Citation: Ma ZA, Wang LX, Zhang H, Li HZ, Dong L, Wang QH, Wang YS, Pan BC, Zhang SF, Cui HT, Lv SQ. Jianpi Gushen Huayu decoction ameliorated diabetic nephropathy through modulating metabolites in kidney, and inhibiting TLR4/NF-κB/NLRP3 and JNK/P38 pathways. *World J Diabetes* 2024; 15(3): 502-518
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INTRODUCTION

As a frequent complication of type 2 diabetes mellitus (T2DM), diabetic nephropathy (DN) is insidious and highly prevalent. In the early stages, DN progresses slowly and is reversible; however, in later stages it progresses rapidly and confers an unfavorable prognosis[1]. Conventional treatments of DN include controlling blood glucose levels, reducing blood pressure, and improving microcirculation[2]. The above treatments using Western drugs (*e.g.*, antihypertensives) can prevent DN to a certain extent, however, they exert negligible effects on patients with advanced DN[3]. Therefore, developing safe and reliable agents to cut off or delay the progression of DN has become a well-explored area of research.

Traditional Chinese medicine (TCM) is markedly efficacious in improving the clinical symptoms of DM and delaying its progression[4]. A study speculated that quercetin may be used to treat T2DM by targeting the transduction of mitogen-activated protein kinase (MAPK) pathways[5], and that crocin may be used to treat DM by inducing insulin sensitivity, improving insulin signal transduction, and preventing pancreatic β cell failure[6]. Huanglian Jiedu Decoction may be used to treat T2DM and its complications by synergistically regulating and participating in multiple biological processes (*e.g.*, signal transduction, inflammatory responses, and apoptotic and vascular processes) and pathways[7].

Metabolomics is commonly used in understanding both the interactions between metabolites and pathological conditions and the changes to metabolic profiles during drug interventions[8]. A study wherein the early DN rat sera were quantitatively measured by metabolomics suggested that guanosine, oleic acid, and glutamate may be potential biomarkers of kidney injury[9]. Furthermore, *Scutellaria baicalensis* and *Coptis chinensis* regulated the contents of

trihydroxytrimethyloxindole, leukotrienes, leucylproline, and estradiol in the feces of T2DM rats and primarily interfered with the metabolism of sphingolipids and fatty acids[10-12]. Regulating metabolism to ameliorate DN has recently become a well-explored research area[13]. In another study, metabolomic analysis revealed that the lipid metabolites were improved in the sera of DM mice treated with *Rehmannia glutinosa* and *Coptis chinensis* as compared to that of model mice[14]. Moreover, when the kidney tissue samples of rats were analyzed by metabolomics, orally administered astragaloside IV could protect the kidneys by improving region-specific metabolic disorders[15].

Jianpi Gushen Huayu Decoction (JPGS) - consisting of *Astragalus membranaceus*, *Panax ginseng*, *Comus officinalis*, *Dioscorea opposita*, *Gordon Euryale* seeds, *Rosa laevigata*, *Atractylodes macrocephala*, *Angelica sinensis*, *Salvia miltiorrhiza*, *Rhizoma Chuanxiong*, *Whitmania pigra Whitman*, and *Rhei Radix et Rhizoma* is clinically highly efficacious on DN[16]; nevertheless, its reno-protective mechanism of action on DN remains uninvestigated. In this study, we investigated the therapeutic effect of JPGS in a DN mouse model. We then examined the effect of JPGS on the levels of renal endogenous metabolites by using non-targeted metabolomics. Furthermore, we examined the effects of JPGS on c-Jun N-terminal kinase (JNK)/P38-mediated apoptosis and inflammatory responses as mediated by the toll-like receptor 4 (TLR4)/nuclear factor-kappa B (NF-κB)/NOD-like receptor family pyrin domain containing 3 (NLRP3).

MATERIALS AND METHODS

Animals and materials

Sixty healthy male C57BL/6 mice (with an average body weight of 21 ± 1 g) were obtained from Beijing Huafukang Co., Ltd. Each cage which was maintained at a room temperature of 20-23 °C and at relative humidity levels of 50%-60% - housed five mice which were entrained to a 12 h/12 h light-dark cycle. The mice had free access to food and water. The animal study was reviewed and approved by Ethics Committee of Hebei University of Chinese Medicine (Approval No. CZX2021-KY-026). Details of the materials and reagents employed in this study were shown in Supplementary material.

Preparation of JPGS

In accordance with the JPGS prescription, the following quantities of single extract of Chinese traditional medicinal crops were weighed: 7 g of *Astragalus membranaceus*, 2.1 g of *Panax ginseng*, 4.4 g of *Comus officinalis*, 1.8 g of *Dioscorea opposita*, 0.7 g of *Gordon Euryale* seeds, 2.4 g of *Rosa laevigata*, 4.1 g of *Atractylodes macrocephala*, 3.5 g of *Angelica sinensis*, 2.6 g of *Salvia miltiorrhiza*, 1.8 g of *Rhizoma Chuanxiong*, 0.4 g of *Whitmania pigra Whitman*, and 1.2 g of *Rhei Radix et Rhizoma*. The TCM prescription dispensing machine (ARTEMIS-M540, EFong Pharmaceutical, Guangdong, China) was used to combine the above extracts into a single bag for further use. Subsequently, the mixed extracts were dissolved with water, and the concentration was controlled to 5.3 g/mL. The mixture was stored at 4 °C. Ultra performance liquid chromatography coupled with mass spectrometer was conducted as the quality control of JPGS, as detailed in the Supplementary material. Total ion chromatogram and chromatography of JPGS were shown in Supplementary Figure 1 and Supplementary Table 1.

Animal experiments

All mice underwent one-week adaptive feeding before receiving a high-sugar and high-fat diet (HFD) containing 34% sucrose, 21% fat, 0.15% cholesterol, and 44.85% conventional treats for eight weeks. After eight weeks of HFD feeding, the mice were modeled as follows: All mice underwent fasting for 12 h with unrestricted access to water and were then intraperitoneally treated with 30 mg/kg streptozotocin (STZ), except for the mice in the control group, which were intraperitoneally administrated with an equal volume of the vehicle. Seventy-two hours following STZ administration, blood from the tail vein was collected from the mice to perform fasting blood glucose (FBG) tests, and mice with a FBG ≥ 12.0 mmol/L were confirmed as having T2DM. The mice were then fed with HFD continuously and tested weekly for 24 h urine total protein (24 h-UTP). Successful DN modeling was identified based on the criteria of a FBG ≥ 12.0 mmol/L and a 24 h-UTP ≥ 20 mg.

After modeling; the mice were divided into the model, irbesartan (IRBE), low-dose JPGS (JPGSL), medium-dose JPGS (JPGSM), and high-dose JPGS (JPGSH) groups according to the random number table, with ten mice assigned to each group. The mice, in the control and model groups received 0.2 mL of normal saline; in the IRBE group received 30 mg/kg/d IRBE; and in the JPGSL, JPGSM, and JPGSH groups received 2.4, 4.8, and 9.6 g/kg/d, *via* gavages, respectively. The duration of administration in each group was four consecutive weeks, and FBG levels and body weight were measured weekly. After four weeks of JPGS treatment, 24 h urinary samples of the mice in each group were collected in metabolic cages. In addition, blood samples were collected *via* the inner canthus. After the mice were euthanized, the abdominal cavity was opened; the left kidneys were collected and immobilized in a 4% paraformaldehyde solution, whereas the right kidneys were cryopreserved.

Measurement of kidney function, oxidative stress, and inflammation-related parameters

The collected 24 h urinary samples were centrifuged (4000 × *g*, 10 min) to obtain the supernatant. 24 h-UTP was measured according to the kit instructions. Blood samples were centrifuged (400 × *g*, 15 min) to prepare serum, while the creatinine (Cr) and blood urea nitrogen (BUN) levels in sera were measured according to the kit instructions. Frozen kidney tissue samples and normal saline were mixed at a 1:9 ratio, the resulting mixture was homogenized and centrifuged (400 × *g*, 15 min), and the supernatant was prepared for the kidney tissue homogenate. The activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and the level of malondialdehyde (MDA) in the kidney tissue homogenates were

measured according to the kit instructions; the levels of interleukin (IL)-6, IL-1β, and tumor necrosis factor alpha (TNF-α) in the kidney tissue homogenates in each group were detected by enzyme-linked immunosorbent assay. Meanwhile, total protein concentrations in kidney tissue homogenates were tested to homogenize the samples.

Hematoxylin and eosin, periodic acid Schiff, and TUNEL staining

Kidneys immobilized in a 4% paraformaldehyde solution were dehydrated, embedded in paraffin, and sliced into 4 μm-thick sections, which sequentially underwent hematoxylin and eosin (HE), periodic acid Schiff (PAS), and TUNEL staining. The dehydrated and mounted sections were then microscopically observed. Quantification of HE staining was conducted according to the severity of tubular dilatation, brush border loss, tubular necrosis, and cast formation as established in a previous study[17]. Moreover, the glomerulosclerosis index was used to assess the severity of glomerulosclerosis in PAS staining[18]. Apoptosis was observed using TUNEL staining, and TUNEL staining-positive regions were measured by Image J (version 1.52a, NIH, Bethesda, MD, United States).

Non-targeted metabolomics analysis

A total of 100 mg of frozen kidney tissue was finely ground in liquid nitrogen, placed into a 1.5 mL tube with 500 μL of 80% methanol; the tube was then vortexed, oscillated, and cooled. Further, the mixture was centrifuged (15000 × *g*, 20 min). The supernatant was obtained and diluted with ultrapure water to a volume containing 53% methanol, which was centrifuged at 15000 × *g* at 4 °C for 20 min to remove the supernatant. An aliquot (20 μL) of each sample was obtained; all the aliquots were subsequently mixed well. The resulting mixture was taken as the quality control sample. See Supplementary material for details of liquid chromatography-mass spectrometry conditions, gradient elution program (Supplementary Table 2) and data acquisition, processing, and steps taken in conducting the analysis.

Western blotting

Approximately 30 mg of frozen kidney tissue was weighed so as to extract the total protein available. The concentrations of the protein samples were then investigated, and a loading buffer was mixed with protein samples, which underwent denaturation *via* incubation at 97 °C for 5 min. The protein samples were separated using sodium-dodecyl sulfate gel electrophoresis and transferred onto a 0.22 μm polyvinylidene fluoride membrane using the wet transfer method. After 2 h blocking in 5% skim milk, TLR4, P65, phosphorylated P65 (p-P65), NLRP3, cleaved-caspase-1, caspase-1, protein apoptosis-associated speck-like protein containing a CARD (ASC), IL-1β, IL-18, phosphorylated JNK (p-JNK), JNK, phosphorylated p38 (p-P38), P38, caspase-3, caspase-9, and β-actin were added respectively for the primary antibody incubation at 4 °C overnight. Further, the membranes were washed and the corresponding secondary antibodies, diluted at a volume ratio of 1:4000, were added and incubated for 2 h at room temperature. The membranes were then washed thrice. After adding an enhanced chemiluminescence developer for a complete reaction; the membranes were exposed to a stand-alone gel imaging system so as to reveal the protein bands. ImageJ software was utilized to measure the grey values of various bands and to quantify the relative expression of the proteins.

Reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted from the kidney tissue using Trizol reagent, followed by reverse transcription into cDNA using a kit. Gene expression was analyzed using a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) detection system and a SuperReal PreMix Plus kit. At the messenger ribonucleic acid (mRNA) level, the expression quantity of target genes relative to the housekeeping gene *Actb* was calculated *via* the $2^{-\Delta\Delta CT}$ method. Primer sequences were shown in Supplementary Table 3.

Statistical methods

SPSS Statistics 20.0 was used for data analysis. All data were presented as mean ± SD. A one-way analysis of variance was conducted for multi-group comparisons and Tukey's honest significance difference test was used for *post-hoc* analysis, while the inter-group pairwise comparisons were conducted using a two-tailed Student's *t*-test. Analysis results with a P value < 0.05 were considered statistically significant.

RESULTS

Ameliorative effects of JPGS in a DN mouse model

During the four-week treatment period, alterations in the body weight and FBG levels were dynamically recorded on a weekly basis. After JPGS administration, body weight decreased significantly, and the FBG levels increased remarkably in DN mice compared to that of the normal mice. The mice's body weight increased and the FBG levels decreased notablyin the IRBE, JPGSL, JPGSM, and JPGSH groups in comparison with that of the mice in the model group (Figure 1A and B). The kidney function test results revealed that the Cr and BUN levels and 24 h-UTP increased considerably in the mouse sera in the model group than that those of the control group. Furthermore, the Cr level markedly reduced in the IRBE, JPGSL, JPGSM, and JPGSH groups. The BUN level and 24 h-UTP decreased notably in the IRBE and JPGSH groups when compared to that of the model group (Figure 1C-E). The HE and PAS staining results showed that severe pathological changes (*e.g.*, glomerular hypertrophy, hyperplasia of mesangial matrix, and thickened glomerular basement membrane) occurred in the kidneys in the model group and could be improved by IRBE and JPGS (Figure 2A-D).

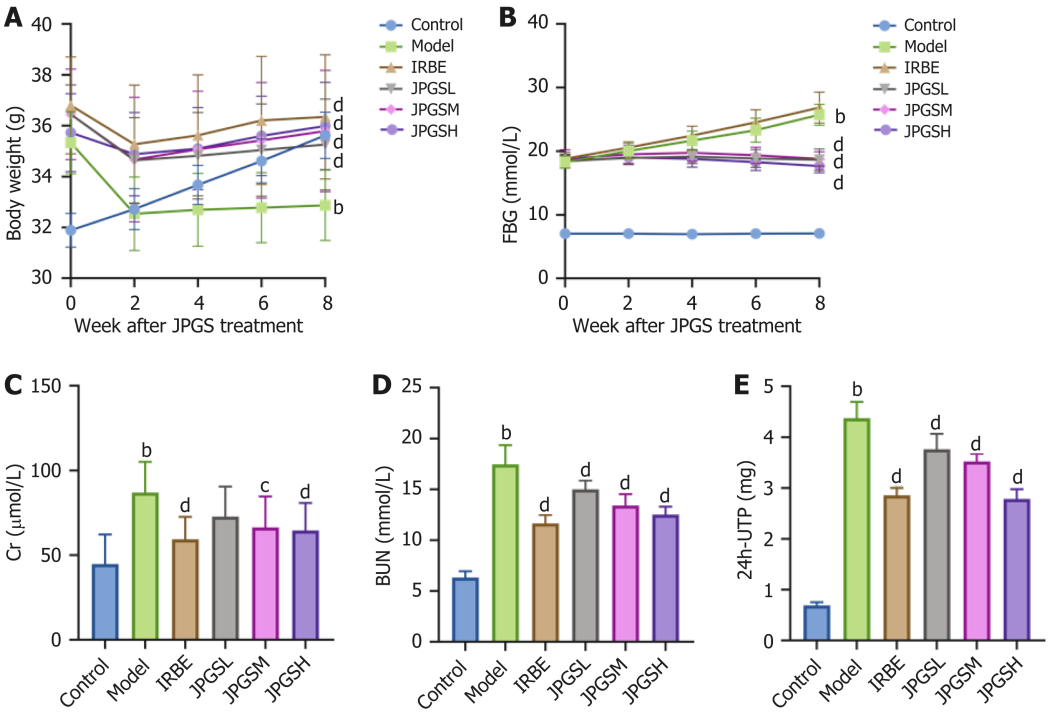

Figure 1 Jianpi Gushen Huayu Decoction administration improved body weight loss, hyperglycemia, and kidney injury in diabetic nephropathy mice. A: Curves of body weight changes; B: Curves of fasting blood glucose changes; C: Creatinine levels; D: Blood urea nitrogen level; E: 24 h urine total protein levels. Control, model, irbesartan, low-dose Jianpi Gushen Huayu Decoction (JPGSL), medium-dose JPGS, and high-dose JPGS groups, n = 10 per group. $^{b}P < 0.01$ as compared to the control group; $^{c}P < 0.05$ as compared to the model group; $^{d}P < 0.01$ as compared to the model group. JPGS: Jianpi Gushen Huayu Decoction; FBG: Fasting blood glucose; Cr: Creatinine; BUN: Blood urea nitrogen; 24h-UTP: 24 h urine total protein; JPGSL: Low-dose Jianpi Gushen Huayu Decoction; JPGSM: Medium-dose Jianpi Gushen Huayu Decoction; JPGSH: High-dose Jianpi Gushen Huayu Decoction; IRBE: Irbesartan.

Changes in oxidative stress and inflammatory factor levels in DN mice following JPGS treatment

The activities of anti-oxidative enzymes (SOD, GSH-Px) decreased substantially, whereas the MDA level (a product of lipid peroxidation) notably increased in the model group in comparison to the control group, suggesting that oxidative stress was exacerbated in the kidney tissue of DN mice. The SOD and GSH-Px levels were markedly elevated and the MDA level decreased dramatically in IRBE, JPGSL, JPGSM, and JPGSH groups when compared to that of the model group, indicating that JPGS could notably alleviate oxidative stress in DN mice (Figure 3A-C).

The IL-6, IL-1β, and TNF-α levels increased significantly in the model group compared to those of the control group and decreased noticeably in the IRBE, JPGSL, JPGSM, and JPGSH groups when compared to those of the model group, demonstrating that JPGS can notably reduce the inflammatory levels observed in the kidneys of DN mice (Figure 3D-F).

Effects of JPGS on metabolites in kidney tissues of DN mice

When examining the effects of JPGS on treating DN and improving oxidative stress, JPGSH had the highest improvement effect in DN mice. Therefore; control, model, and JPGSH groups were selected in this study to conduct metabolomics analyses. The principal component analysis results showed that the distributed clusters were markedly separated, signaling that the DN mice in the model group were successfully modeled, and that JPGS could considerably improve DN-associated kidney metabolite abnormalities in DN mice (Figure 4A). A predictive model was established by the partial least squares discriminant analysis (PLS-DA) and validated to further define inter-group differences. Based on a PLS-DA prediction model, we observed significant separation of the control and model groups, as well as the model and JPGSH groups, on the PLS-DA score maps with R2Y values in both cases being greater than 0.9, and Q2 values being less than zero. This indicates that the model did not overfit and that the results carried a high degree of explanatory power (Figure 4B-E).

An ionic variable importance in projection score > 1, $P < 0.05$, and a fold change > 2 or < 0.5 were the screening criteria for differential metabolites. A total of 51 differential metabolites were screened out from the control, model, and JPGSH groups (Table 1). The screened differential metabolites were introduced into MetaboAnalyst for metabolic pathway analysis. A total of four metabolic pathways: (1) Cysteine and methionine metabolism; (2) Alanine, aspartate and glutamate metabolism; (3) Tryptophan metabolism; and (4) Riboflavin metabolism were enriched as the common pathways (Figure 4F and G).

Changes in the TLR4/NF-κB/NLRP3 pathway in the kidney tissue of DN mice following JPGS treatment

Recent studies suggested that the tryptophan metabolite N-acetylserotonin (NAS) could inhibit inflammatory responses *via* the TLR4/NF-κB/NLRP3 pathway[19]. Our findings revealed that JPGS could reduce the NAS level in kidney tissue. As such, we evaluated the effect of JPGS on the TLR4/NF-κB/NLRP3 pathway. Specifically, we measured the levels of

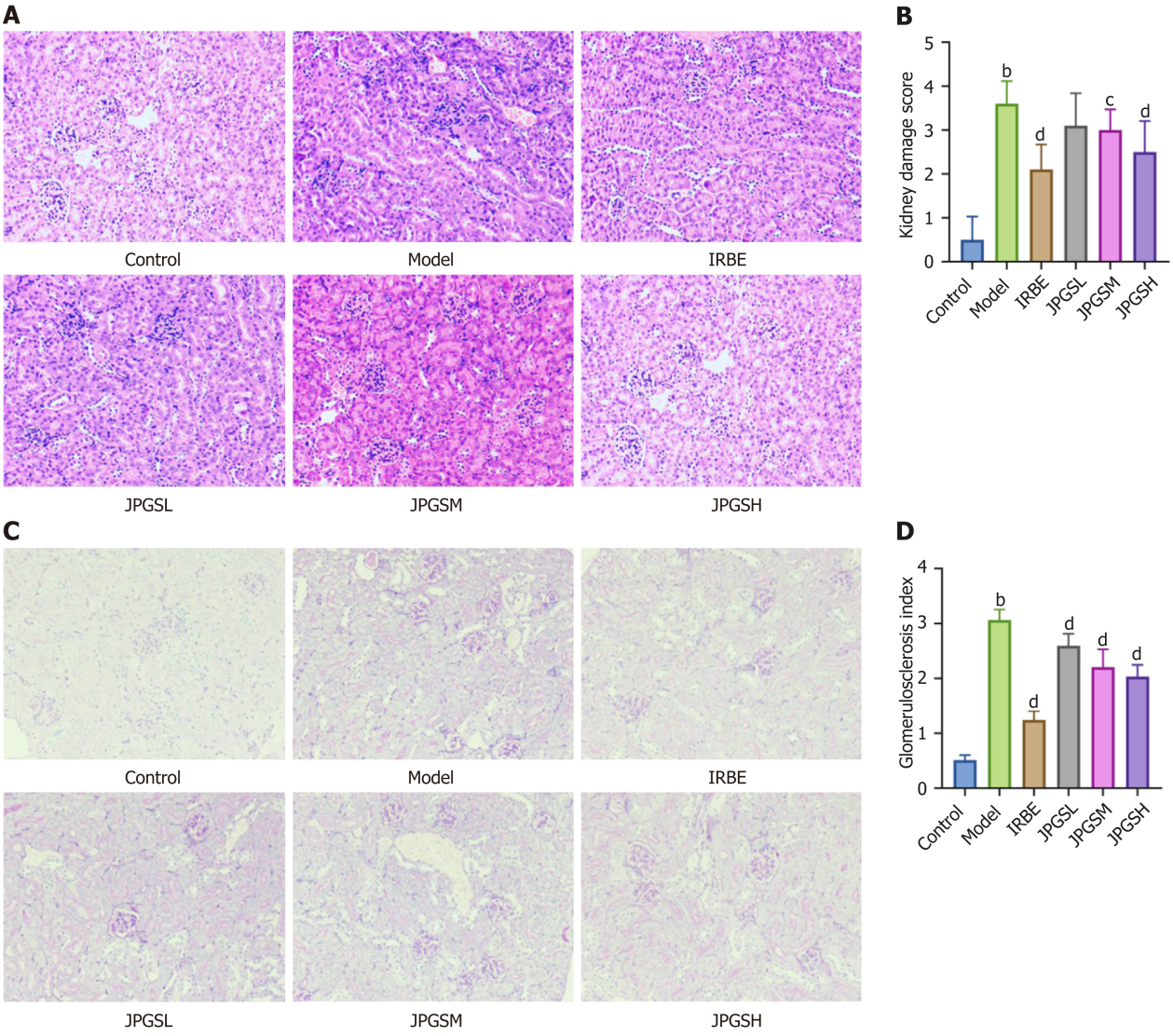

Figure 2 Pathological changes in kidneys were improved following Jianpi Gushen Huayu Decoction administration. A: Hematoxylin and eosin staining; B: Kidney damage score; C: Periodic acid Schiff staining; D: Glomerulosclerosis index, magnification × 100. $^{b}P < 0.01$ as compared to the control group; $^{c}P < 0.05$ as compared to the model group; $^{d}P < 0.01$ as compared to the model group. JPGSL: Low-dose Jianpi Gushen Huayu Decoction; JPGSM: Medium-dose Jianpi Gushen Huayu Decoction; JPGSH: High-dose Jianpi Gushen Huayu Decoction; IRBE: Irbesartan.

the critical proteins (*i.e.,* TLR4, p-P65/P65, NLRP3, ASC, cleaved caspase-1, mature IL-1β, and IL-18) in the TLR4/NF-κB/NLRP3 pathway using western blotting. The results signified that the expression levels of these critical proteins were notably elevated in the mouse kidneys in the model group compared to that of the control group, and decreased notably after treatment with JPGSH (Figure 5A-H).

Additionally, we measured the expression levels of *Nlrp3*, *Asc*, *casp1*, *IL1b*, and *Il18* in the TLR4/NF-κB/NLRP3 pathway using quantitative RT-qPCR, with the results indicating that, at the mRNA level, the expression levels of *Nlrp3*, *Asc*, *casp1*, *IL1b*, and *Il18* increased notably in the mouse kidneys of the model group when compared to those of the control group and decreased significantly after treatment with JPGSH (Figure 5I-M).

Effect of JPGS on JNK/P38-mediated apoptosis in DN mice

A previous study has indicated that 5-hydroxyindole-3-acetic acid (5-HIAA), a tryptophan metabolite, could promote cell apoptosis *via* the JNK/P38 pathway[20]. The metabolomics results revealed that JPGS could reduce the 5-HIAA level. Therefore, we examined whether JPGS could affect JNK/P38 pathway-mediated apoptosis. Specifically, we observed the effect of JPGSH on apoptosis in DN mice using TUNEL staining; the results indicated that a massive amount of green fluorescence could be found in the kidney tissue of DN model mice, and positive fluorescence was markedly diminished in the kidney tissue of DN mice following JPGSH administration (Figure 6A and B).

Furthermore, we tested the levels of JNK phosphorylation, P38 phosphorylation, cleaved caspase-3, and cleaved caspase-9 in the JNK/P38 pathway; the results indicated that the degrees of p-JNK/JNK, p-P38/P38, cleaved caspase-3, and cleaved caspase-9 increased remarkably in the mouse kidneys of the model group when compared to those of normal mice and decreased dramatically after treatment with JPGSH (Figure 6C-G).

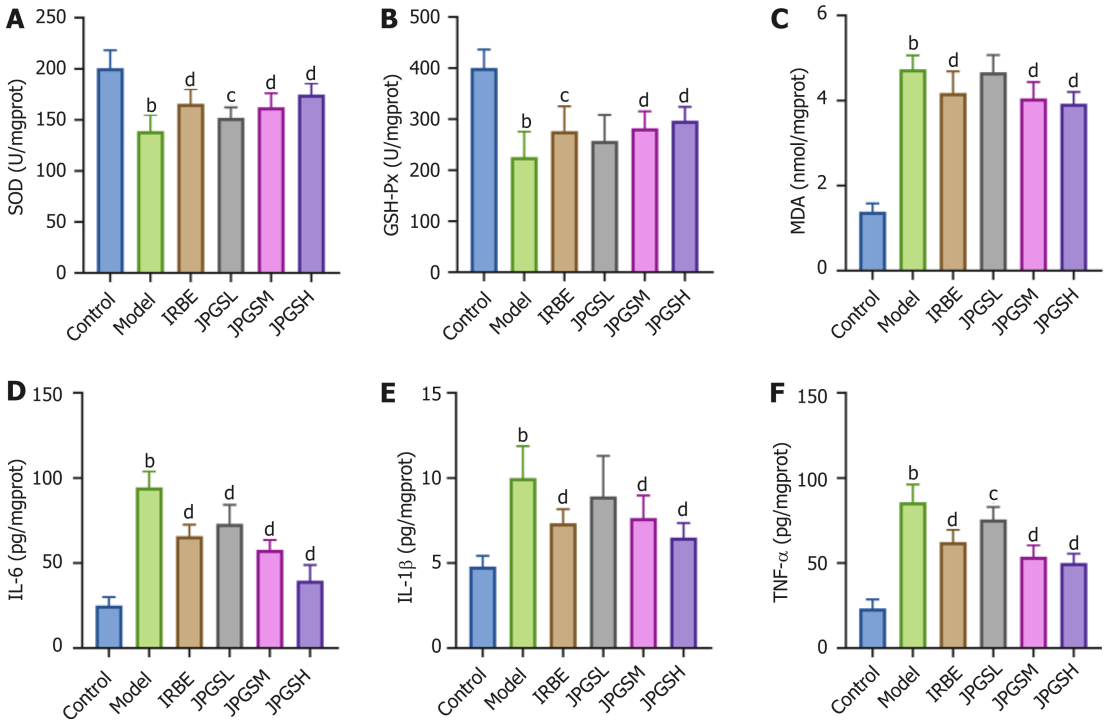

Figure 3 Jianpi Gushen Huayu Decoction reduced oxidative stress and inflammation in kidneys. A: The activities of superoxide dismutase; B: The activities of glutathione peroxidase; C: Malondialdehyde level; D: Interleukin (IL)-6 levels; E: IL-1β levels; F: Tumor necrosis factor-α levels. $^{b}P < 0.01$ as compared to the control group; $^{c}P < 0.05$ as compared to the model group; $^{d}P < 0.01$ as compared to the model group. JPGSL: Low-dose Jianpi Gushen Huayu Decoction; JPGSM: Medium-dose Jianpi Gushen Huayu Decoction; JPGSH: High-dose Jianpi Gushen Huayu Decoction; IRBE: Irbesartan; SOD: Superoxide dismutase; GSH-Px: Glutathione peroxidase; MDA: Malondialdehyde; IL-6: Interleukin 6; IL-1β: Interleukin 1 beta; TNF-α: Tumor necrosis factor alpha.

DISCUSSION

JPGS, whose mechanism of action remains unknown, is commonly employed to treat DN in clinical practice[16]. In this study, we analyzed the effect of JPGS on the metabolism of DN mice and investigated the therapeutic effect of JPGS on DN and its underlying mechanism of action by using non-targeted metabolomics. The classic model mice with DN, as induced by HFD combined with STZ, could exhibit similar symptoms to those observed in patients with DN[21]. In this model, the mice were fed a high-sugar diet to induce insulin resistance and were injected with STZ to induce pancreatic β cell necrosis to trigger the loss of the insulin secretion function. As a result, persistent hyperglycemia led to kidney tubular atrophy, necrosis, and nephrotoxicity resulting in impaired kidney function and organic injury[22]. 24 h-UTP, which is the most sensitive parameter to assess early DN, is indicative of glomerular filtration impairment[23]. Cr and BUN levels, which are the major parameters used to assess kidney function, are generally utilized to estimate the glomerular filtration rate and an increase in the Cr and BUN levels in the serum signifies kidney dysfunction[24,25]. In this study, severe pathological changes (*e.g.*, glomerular and kidney tubular epithelial cell hypertrophy and thickened basement membrane), corresponding to the above symptoms occurred in the kidneys of DN mice and were effectively improved by JPGS. Moreover, IRBE is frequently employed to treat DN in clinical practice which was taken as the positive control in this study[26]. The findings revealed that JPGSH and IRBE were not significantly different in terms of efficacy. This suggested that JPGS could markedly treat DN; however, unveiling its mechanism of action which remains unknown requires further investigation.

Oxidative stress and inflammatory responses contribute considerably to the occurrence and progression of DN. Oxidative stress can induce inflammatory responses which, in turn, can exacerbate oxidative stress, while inflammation and oxidative stress can lead to alterations in kidney structure and function *via* multiple pathways[27]. In this study, JPGS exerted antioxidant and anti-inflammatory effects by helping to produce SOD and GSH-Px and reducing the MDA and proinflammatory cytokine contents. The exacerbation of oxidative stress is associated with an increase in the reactive oxygen species level, which correlates with endothelial dysfunction, changes in the extracellular matrix protein level, and an increase in kidney sodium reabsorption levels[28-30]. Exacerbated oxidative stress in DM patients could contribute to the occurrence of DN and cause DN to progress to end-stage kidney disease[31]. SOD and GSH-Px, which are major antioxidant enzymes, play a crucial role in maintaining the oxidant/antioxidant balance, whereas MDA is the lipid peroxidation end product in the body and its content is indicative of the degree of cellular oxidative injury[32]. IL-6, IL-1β, and TNF-α, which are common inflammatory factors, play a vital role in inflammatory responses in the body[33]. Persistent hyperglycemia led to continuously elevated expression levels of inflammatory factors, resulting in exacerbated inflammatory responses[34].

The above studies on the efficacy of JPGS proved that it can effectively treat DN and that JPGSH had the greatest effect on improving DN. Thus, we selected the control, model, and JPGSH groups to investigate potential therapeutic pathways

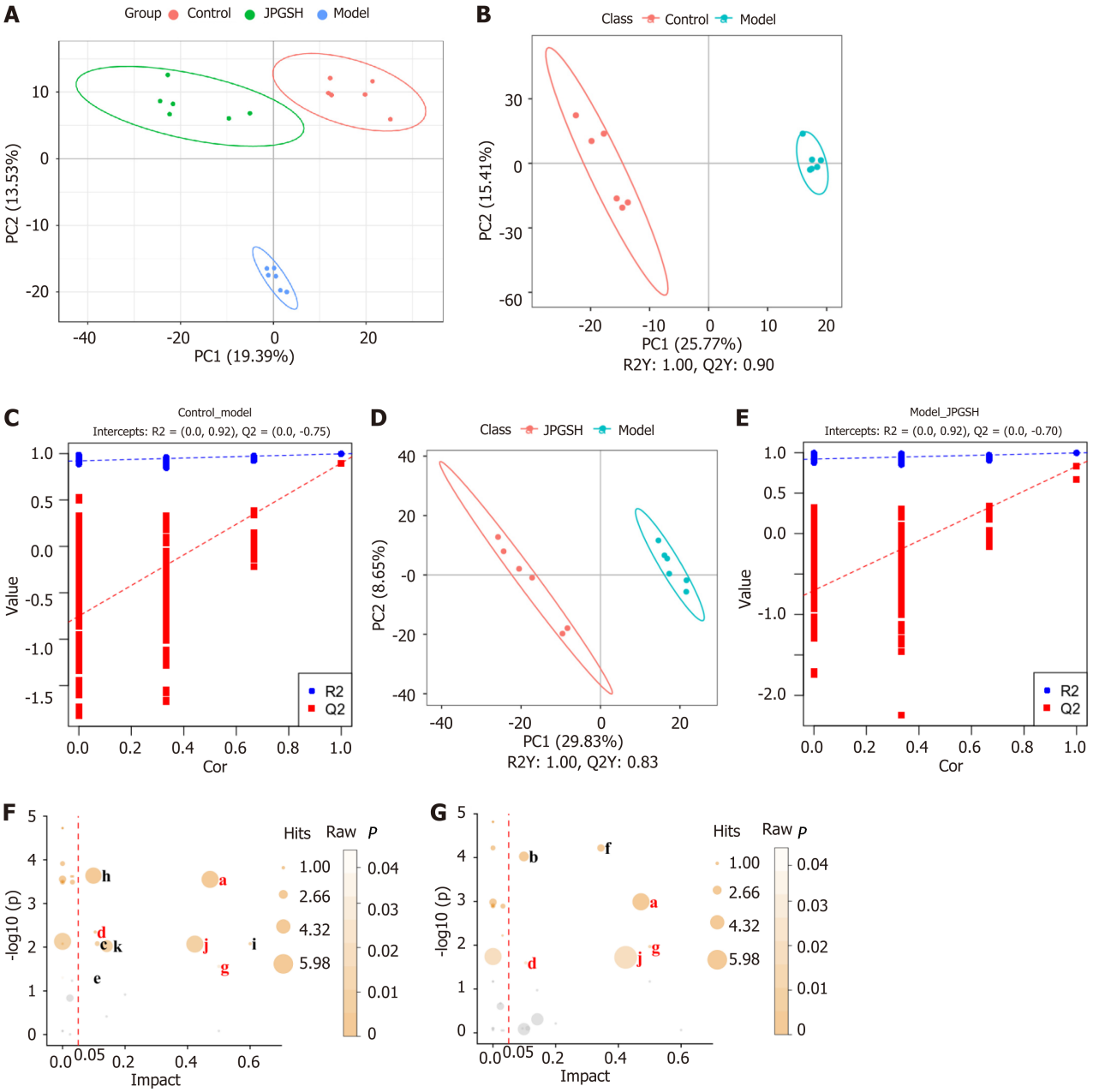

Figure 4 Jianpi Gushen Huayu Decoction treatment alters the metabolites in kidneys. A: Score plots of PCA among control, model, and high-dose Jianpi Gushen Huayu Decoction (JPGSH) groups; B and C: Score plots and permutation tests of partial least squares discriminant analysis (PLS-DA) between the control and model groups; D and E: Score plots and permutation tests of PLS-DA between the model and JPGSH groups; F: Results of the pathway analysis between the control and model groups; G: Results of the pathway analysis between the model and JPGSH groups. Common pathways are marked in red. The names of the pathways are represented by "a" to "k": a: Alanine, aspartate and glutamate metabolism; b: Arachidonic acid metabolism; c: Butanoate metabolism; d: Cysteine and methionine metabolism; e: Glycerophospholipid metabolism; f: Histidine metabolism; g: Riboflavin metabolism; h: Steroid hormone biosynthesis; i: Synthesis and degradation of ketone bodies; j: Tryptophan metabolism; k: Tyrosine metabolism. *n* = 6 per group. JPGSH: High-dose Jianpi Gushen Huayu Decoction.

by conducting metabolomics analyses. The results revealed that alanine, aspartate, and glutamate metabolism; cysteine and methionine metabolism; riboflavin metabolism; and tryptophan metabolism were the common metabolic pathways, signaling that the above pathways potentially functioned for treating DN.

The findings thus revealed that JPGS could regulate glucose metabolism and alanine, aspartate, and glutamate levels, which may be the underlying mechanism of ameliorating DN. Alanine, aspartate, and glutamate which are non-essential amino acids in humans, play a pivotal role in glycolysis and the tricarboxylic acid cycle; furthermore, their metabolism correlates closely with the progression of DM[35,36]. A study has suggested that alanine, aspartate, and glutamate levels in the sera of patients with DM as complicated by obesity were elevated and that glucose metabolism disorders induced by alanine, aspartate, and glutamate metabolism disorders potentially facilitated the progression of DM[37]. Alanine, a non-essential amino acid synthesized from pyruvate and branched chain amino acids, can increase glucose-dependent insulin secretion by inducing membrane depolarization during its co-transport with sodium ions[38]. Glutamate, synthesized from α-ketoglutaric acid produced by the deamination of glutamate under the action of glutaminase, can be oxidized to produce energy for the citric acid cycle independently of glucose[39] and to produce adenosine triphosphate

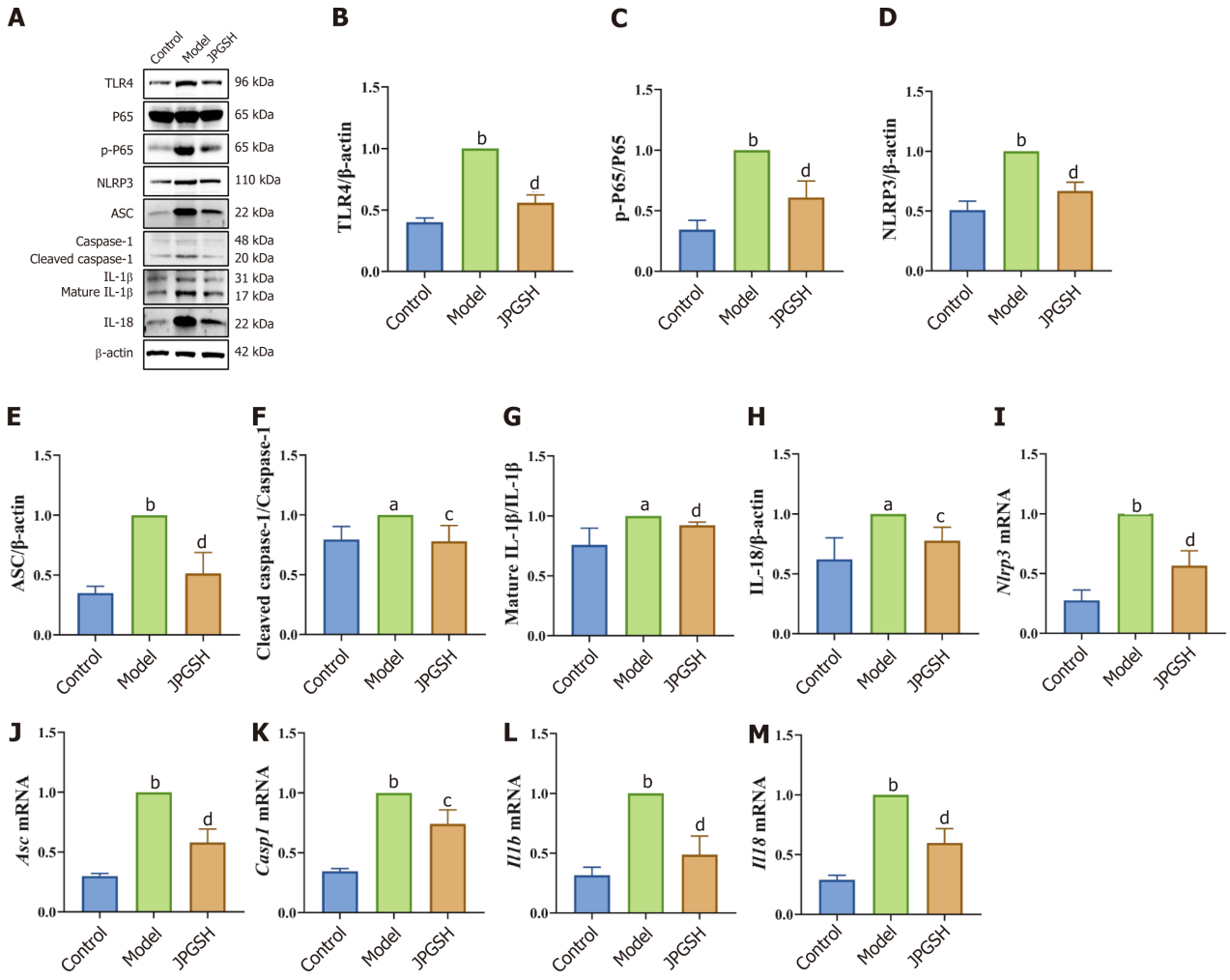

Figure 5 Jianpi Gushen Huayu Decoction moderated the toll-like receptor 4/nuclear factor-kappa B/NOD-like receptor family pyrin domain containing 3 pathway and ameliorated inflammatory injuries to kidneys. A: Western blotting results; B: Toll-like receptor 4 levels; C: p-P65/P65 levels; D: NOD-like receptor family pyrin domain containing 3 (NLRP3) levels; E: Apoptosis-associated speck-like protein containing a CARD (ASC) levels; F: Cleaved caspase-1/caspase-1 levels; G: Mature interleukin (IL)-1β/IL-1β levels; H: IL-18 levels; I: *Nlrp3* levels; J: *Asc* levels; K: *Casp1* levels; L: *Il1b* levels; M: *Il18* levels. $^{a}P < 0.05$ as compared to the control group; $^{b}P < 0.01$ as compared to the control group; $^{c}P < 0.05$ as compared to the model group; $^{d}P < 0.01$ as compared to the model group. JPGSH: High-dose Jianpi Gushen Huayu Decoction; TLR4: toll-like receptor 4; NLRP3: NOD-like receptor family pyrin domain containing 3; ASC: Protein apoptosis-associated speck-like protein containing a CARD; IL: Interleukin.

to stimulate insulin secretion[38]. Additionally, a high level of glutamate can induce pancreatic β cell apoptosis, worsening DM[40]. Aspartate, produced by the hydrolysis of asparagine by L-asparaginase, participates in glucose production and maintaining a low level of aspartate in the blood can help reduce glycogen synthesis to diminish the risk of hyperlipidemia[41,42]. The above results indicate that glucose metabolism disorders could facilitate the progression of DM. The correlation between DN and alanine, aspartate, and glutamate metabolism remains scarcely investigated to date, and determining the underlying mechanism of JPGS requires further study.

In this study, JPGS could up-regulate the methionine level to regulate the methionine cycle and balance the *in vivo* homocysteine (Hcy) level to lessen kidney injury in DN mice. Cysteine and methionine are sulfur-containing amino acids. Cysteine is formed from serine and the condensation product of methionine-derived Hcy is used as the sulfur source. Methionine is an essential amino acid which animals cannot synthesize. Hcy, an intermediate metabolite of cysteine and methionine, plays a vital role in the progression of DN[43]. Hcy is a sulfur-containing non-essential amino acid and healthy kidneys are essential in Hcy clearance and metabolism; since the Hcy-specific tubular uptake mechanism and the metabolic enzymes of Hcy are present in the kidneys[44]. Studies showed that Hcy is a factor that affects urinary protein excretion independently of hypertension, DM, protein intake, and kidney function[45]. DN may be complicated by hyperhomocysteinemia (HHcy), whose pathogenesis is principally associated with the *in vivo* accumulation of Hcy caused by impaired glomerular filtration and Hcy metabolism disorder caused by the impaired integrity of the kidney structure and function. In addition, HHcy may exacerbate insulin resistance to contribute to the occurrence and progression of DN and HHcy and DN have a mutually exacerbating effect on each other[46]. The potential mechanism of treating DN by regulating cysteine and methionine metabolism must be studied further.

Our results indicated that JPGS could markedly up-regulate the riboflavin level in the kidney tissues of DN mice. Riboflavin, an essential nutrient for humans and animals, is a major food additive. Riboflavin performs critical metabolic functions by mediating electron transfer in biological redox reactions and participates in the metabolism of folate, vitamin

Table 1 Differential metabolites in kidney after Jianpi Gushen Huayu Decoction treatment

No.	Metabolites	Formula	RT (min)	m/z	FC		VIP		Trend		Pathway
					M *vs* C	J *vs* M	M *vs* C	J *vs* M	M *vs* C	J *vs* M	
1	15(S)-HpETE	C20H32O4	12.78	335.22	1.14	0.45	0.35	1.71	↑	↓[d]	Arachidonic acid metabolism
2	16(R)-HETE	C20H32O3	13.91	343.22	1.23	0.28	0.58	1.69	↑	↓[d]	Arachidonic acid metabolism
3	1-naphthol	C10H8O	8.86	145.06	3.00	0.53	1.51	1.19	↑[b]	↓[c]	
4	2-isopropylmalate	C7H12O5	1.32	175.06	1.97	2.06	1.61	1.37	↑[b]	↑[c]	
5	2-methoxyresorcinol	C7H8O3	11.65	141.05	1.21	1.20	1.37	1.34	↑[a]	↑[c]	
6	3-hydroxy-3-methyl-glutaric acid	C6H10O5	1.32	161.05	1.68	1.72	1.42	1.33	↑[a]	↑[c]	
7	3-indoleacrylic acid	C11H9NO2	6.03	186.06	0.52	1.63	1.83	1.38	↓[b]	↑[c]	
8	4-guanidinobutyric acid	C5H11N3O2	1.35	129.07	0.42	1.67	1.53	1.38	↓[b]	↑[c]	Butanoate metabolism
9	4-hydroxybenzoic acid	C7H6O3	5.73	137.02	0.57	3.72	1.39	1.65	↓[a]	↑[d]	
10	4-oxoretinol	C20H28O2	13.68	299.20	0.47	1.67	1.42	1.37	↓[a]	↑[c]	
11	5-hydroxyindole-3-acetic acid	C10H9NO3	7.05	192.07	7.50	0.04	1.31	1.44	↑[b]	↓[d]	Tryptophan metabolism
12	5-hydroxytryptophan	C11H12N2O3	6.55	221.09	0.23	2.30	1.50	1.65	↓[b]	↑[d]	Tryptophan metabolism
13	ACar 20:1	C27H52NO4	13.89	472.40	1.86	0.49	1.67	1.63	↑[b]	↓[d]	
14	Acetoacetate	C4H6O3	1.44	101.02	2.12	1.55	1.35	1.14	↑[a]	↑	Arachidonic acid metabolism, synthesis and degradation of ketone bodies, tyrosine metabolism
15	Adrenosterone	C19H24O3	13.34	301.18	1.18	0.67	0.62	1.19	↑	↓[c]	Steroid hormone biosynthesis
16	Alpha-ketoglutaric acid	C5H6O5	1.25	145.01	0.66	2.60	1.69	1.49	↓[b]	↑[d]	Alanine, aspartate and glutamate metabolism, butanoate metabolism
17	Anthranilic acid	C7H7NO2	1.55	138.06	0.71	3.98	1.26	1.83	↓[a]	↑[d]	Tryptophan metabolism
18	Asparagine	C4H8N2O3	1.24	131.05	0.41	5.12	1.33	0.91	↓[b]	↑[c]	Alanine, aspartate and glutamate metabolism
19	Biotin	C10H16N2O3S	8.15	245.10	0.94	2.00	0.21	1.51	↓	↑[d]	
20	Cholesterol	C27H46O	15.23	387.36	0.84	1.51	0.78	1.29	↓	↑[c]	Steroid hormone biosynthesis
21	Citraconic acid	C5H6O4	1.33	129.02	1.36	1.74	1.47	1.57	↑[a]	↑[d]	
22	Citric acid	C6H8O7	6.00	191.02	0.94	1.58	0.19	1.67	↓	↑[d]	Alanine, aspartate and glutamate metabolism
23	Cnidioside A	C17H20O9	1.55	391.10	2.24	3.36	1.32	1.66	↑[a]	↑[d]	
24	Corey lactone diol	C8H12O4	1.30	171.07	1.31	1.91	1.23	1.61	↑[a]	↑[d]	
25	Cortisone	C21H28O5	11.25	361.20	1.85	0.56	1.46	1.44	↑[a]	↓[c]	Steroid hormone biosynthesis
26	D-glucosamine 6-phosphate	C6H14NO8P	1.20	258.04	1.02	0.58	0.10	1.37	↑	↓[c]	Alanine, aspartate and glutamate metabolism
27	D-proline	C5H9NO2	1.54	116.07	0.60	1.97	1.64	1.63	↓[b]	↑[d]	
28	D-threose	C4H8O4	1.31	101.02	1.70	1.76	1.72	1.53	↑[b]	↑[d]	
29	Ecgonine	C9H15NO3	9.72	186.11	3.96	0.51	1.73	1.37	↑[b]	↓[c]	
30	Epitestosterone	C19H28O2	13.23	289.22	1.46	0.32	0.68	1.26	↑	↓[c]	Steroid hormone biosynthesis
31	Estradiol	C18H24O2	14.93	273.18	0.92	0.62	0.34	1.17	↓	↓[c]	Steroid hormone biosynthesis
32	Gamma-tocopherol	C28H48O2	13.26	417.37	2.17	0.36	1.38	1.44	↑[a]	↓[d]	
33	Glutaric acid	C5H8O4	1.43	131.03	1.49	1.93	1.26	1.60	↑[a]	↑[d]	
34	Guanidineacetic acid	C3H7N3O2	1.31	118.06	0.47	1.17	1.71	0.85	↓[b]	↑	
35	Homovanillic acid	C9H10O4	2.09	181.05	0.52	1.40	1.90	1.20	↓[b]	↑	Tyrosine metabolism

36	L-aspartic acid	C4H7NO4	1.21	132.03	1.67	0.35	0.50	1.46	↑[a]	↓[d]	Alanine, aspartate and glutamate metabolism
37	L-glutamine	C5H10N2O3	1.32	147.08	1.39	0.69	1.47	0.31	↑[a]	↓[d]	Alanine, aspartate and glutamate metabolism
38	L-histidine	C6H9N3O2	1.65	154.06	0.60	1.06	1.47	0.30	↓[a]	↑	Histidine metabolism
39	L-kynurenine	C10H12N2O3	8.39	209.09	0.17	3.30	1.60	1.06	↓[b]	↑[a]	Tryptophan metabolism
40	L-thyroxine	C15H11I4NO4	11.98	775.68	0.59	0.79	1.36	0.72	↓[a]	↓	Tyrosine metabolism
41	L-tryptophan	C11H12N2O2	6.70	205.10	0.25	3.24	1.55	1.06	↓[b]	↑[d]	Tryptophan metabolism
42	L-tyrosine	C9H11NO3	1.98	180.07	0.44	1.03	1.95	0.36	↓[b]	↑	Tyrosine metabolism
43	Metanephrine	C10H15NO3	8.92	196.10	3.35	0.21	1.25	1.30	↑[a]	↓[c]	Tyrosine metabolism
44	Methionine	C5H11NO2S	1.43	150.06	0.17	3.10	1.67	1.62	↓[b]	↑[d]	Cysteine and methionine metabolism
45	N-acetylaspartic acid	C6H9NO5	1.21	128.04	1.00	0.54	0.07	1.53	↑	↓[d]	Alanine, aspartate and glutamate metabolism
46	N-acetylserotonin	C12H14N2O2	7.25	219.11	0.29	2.46	1.23	1.44	↓[a]	↑[d]	Tryptophan metabolism
47	Oxoadipic acid	C6H8O5	1.20	159.03	0.54	2.37	1.35	1.52	↓[b]	↑[d]	Tryptophan metabolism
48	PC (16:0/16:0)	C40H80NO8P	16.96	734.57	0.67	1.64	1.18	1.85	↓	↑[d]	Arachidonic acid metabolism, glycerophospholipid metabolism
49	Prostaglandin D2	C20H32O5	11.09	351.22	0.87	0.46	0.36	1.26	↓	↓[c]	Arachidonic acid metabolism
50	Urocanic acid	C6H6N2O2	1.76	139.05	1.61	1.05	1.23	0.54	↑[a]	↑	Histidine metabolism
51	Vitamin B2	C17H20N4O6	7.92	377.15	0.17	4.09	1.48	1.69	↓[b]	↑[d]	Riboflavin metabolism

[a]$P < 0.05$ as compared to the control group.
[b]$P < 0.01$ as compared to the control group.
[c]$P < 0.05$ as compared to the model group.
[d]$P < 0.01$ as compared to the model group.
C: Control group; M: Model group, J: High-dose Jianpi Gushen Huayu Decoction group, $n = 6$ per group; RT: Retention time; VIP: Variable importance for the projection; FC: Fold change.

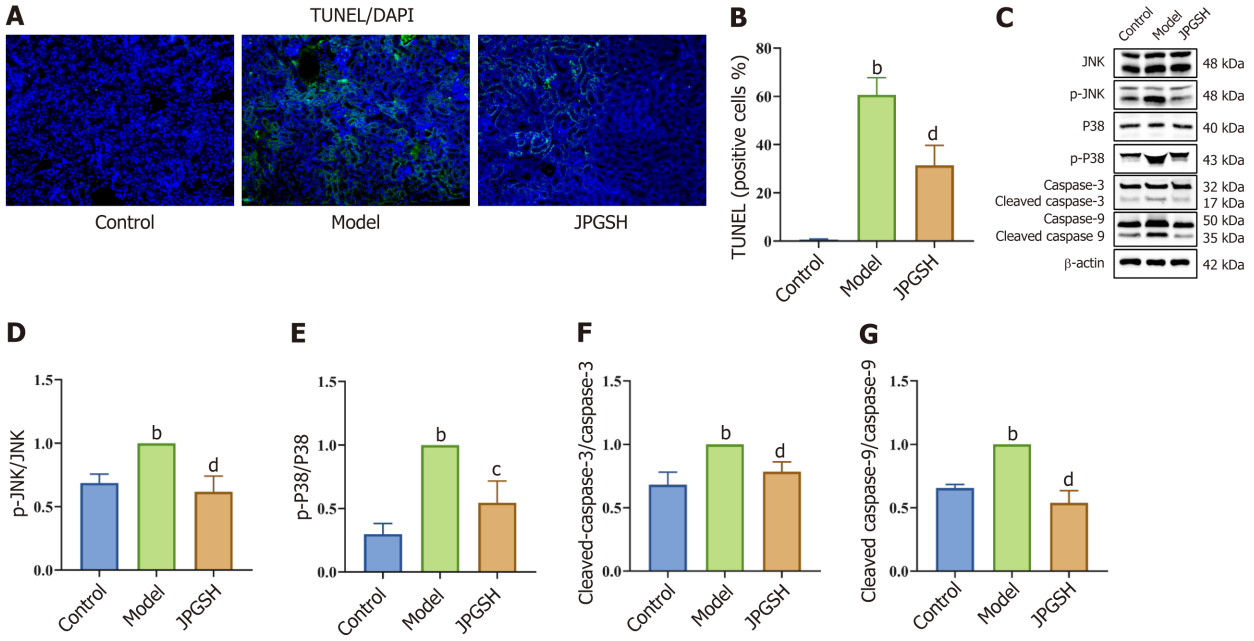

Figure 6 Jianpi Gushen Huayu Decoction inhibited c-Jun N-terminal kinase/P38 pathway mediated apoptosis and improved kidney injury.
A: TUNEL staining; B: Percentage of positive cells in TUNEL staining; C: Western blotting results; D: p-c-Jun N-terminal kinase (JNK)/JNK level; E: p-P38/P38 levels; F: Cleaved-caspase-3/caspase-3 levels; G: Cleaved caspase-9/caspase-9 levels. [b]$P < 0.01$ as compared to the control group; [c]$P < 0.05$ as compared to the model group; [d]$P < 0.01$ as compared to the model group. JPGSH: High-dose Jianpi Gushen Huayu Decoction; JNK: c-Jun N-terminal kinase.

B12, vitamin B6, and other vitamins[47]. Riboflavin, a precursor for flavin mononucleotide and flavin adenine dinucleotide (FAD), serves as a coenzyme for multiple oxidases and dehydrogenases in eucaryotic cells[48]. FAD-dependent Ero1 and sulfhydryl oxidases are involved in secretory proteins oxidative folding, leading to the formation of disulfide bonds[49-52]. Riboflavin deficiency could lead to impaired oxidative folding, resulting in various clinical abnormalities (*e.g.*, kidney injury)[49,50,53]. A study suggested that riboflavin notably inhibited the production of lipid peroxides, enhanced the SOD level, effectively inhibited the connective tissue growth factor (that could significantly exacerbate fibrosis) and showed desirable antioxidant effects in DM rats injected with STZ. This signaled that riboflavin potentially protects against DM-associated systemic fibrosis[54]. Therefore, the effects of JPGS on inhibiting oxidative stress and improving fibrosis in DN mice were possibly related to the regulation of riboflavin metabolism. In future studies, the relationships between the effect of JPGS on regulating riboflavin metabolism and its antioxidant and anti-fibrosis effects should be illuminated.

Furthermore, our findings revealed that tryptophan metabolism disorder occurred in the kidneys of DN model mice; in addition, 5-hydroxytryptophan (5-HTP) and NAS contents decreased to varying degrees, whereas 5-HIAA content increased noticeably. JPGS could restore the levels of the above metabolites. This indicated that JPGS could significantly regulate tryptophan metabolism. Tryptophan, one of the eight essential amino acids in humans, plays a fundamental role in human health and diseases[55]. Tryptophan is principally metabolized *via* 5-hydroxytryptamine (5-HT), kynurenine (KYN), and indole pathways[55]. In the 5-HT pathway, tryptophan is hydroxylated by tryptophan hydroxylase to yield 5-HTP, which is catalyzed by 5-HTP decarboxylase to yield 5-HT. Then, 5-HT reacts with acetyl coenzyme A, as catalyzed by N-acetyltransferase, to form NAS. Recent studies showed that 5-HTP, an effective anti-inflammatory mediator, inhibited the activation of P38 and NF-κB in fibroblasts, reduced the expression levels of inflammatory factors in peripheral blood mononuclear cells[56,57], and inhibited the production of TNF-α induced by lipopolysaccharides to improve inflammatory responses[58,59]. Therefore, JPGS may reduce the TNF-α level by up-regulating the 5-HTP level. A study has reported that 5-HTP could reduce mammary epithelial cell apoptosis in goats *via* the MAPK/extracellular signal-regulated kinase/B-cell lymphoma-3 pathway[60]. NAS, a melatonin precursor, offers anti-inflammatory and antioxidant effects[61]. Another study has suggested that melatonin could mitigate white matter injury in rats subjected to focal ischemia-reperfusion injuries by inhibiting the activation of TLR4/NF-κB pathways, indicating that NAS may regulate the TLR4/NF-κB pathway[62]. Liu *et al*[19] investigated retinal ischemia-reperfusion injury (RIRI) and found that NAS could reduce retinal injuries in rats subjected to RIRI through blocking the activation of the TLR4/NF-κB/NLRP3 pathway to inhibit the expression of IL-1β. 5-HIAA, a metabolic end product of 5-HTP, has been proven to promote apoptosis and activate JNK/P38 pathway-mediated apoptosis[20]. Moreover, tryptophan is the only source of the KYN pathway. In the KYN approach, tryptophan is catabolized into KYN as regulated by indoleamine 2,3-dioxygenase and trypophan-2,3-dioxygenase, and then KYN is metabolized into downstream metabolites (*e.g.*, kynuric, xanthic, and quinolinic acids), which participate in immune activation and inflammation regulation and are associated with obesity and insulin resistance[55,63,64].

Considering the effect of tryptophan metabolism on significantly modulating the TLR4/NF-κB/NLRP3 pathway, we investigated the effect of JPGS on the TLR4/NF-κB/NLRP3 pathway in DN model mice. The study results exhibited that JPGS could restrain the activation of the TLR4/NF-κB/NLRP3 pathway in kidneys. TLR4, a member of the TLR family, plays a crucial role in the cellular antioxidant response and the production of inflammatory cytokines and its activation can lead to NF-κB activation[65-67]. Normally, the NF-κB dimer in the cytoplasm binds to IκBα to form a complex. When IκBα phosphorylation is inactivated, NF-κB is activated and translocated to the nucleus to activate glial cells in order to secrete numerous pro-inflammatory molecules (*e.g.*, IL-1β, IL-18, TNF-α, and IL-6) and induce the expression of the NLRP3 inflammasome[68,69]. The NLRP3 inflammasome is a multi-protein complex composed of NLRP3, ASC, and caspase-1. Activating NLRP3 can promote the assembly of various components (NLRP3, ASC, and caspase-1) of NLRP3 inflammasome to activate pro-caspase-1 to produce active cleaved caspase-1 which, in turn, cleaves and matures the inflammatory factors IL-1β and IL-18. Mature IL-1β can further stimulate the secretion of large quantities of pro-IL-1β, creating a positive feedback loop that amplifies immune responses by activating the NF-κB. As such, DN-related kidney injury can be effectively improved by reducing the activation of the TLR4/NF-κB/NLRP3 pathway[70].

Considering the effects of tryptophan metabolites in markedly regulating JNK/P38-mediated apoptosis, we investigated the effect of JPGS on JNK/P38-mediated apoptosis in DN mice. The study results revealed that JPGS could restrain the activation of the JNK/P38 to reduce kidney cell apoptosis in DN mice. TUNEL staining is commonly employed to assess cell apoptosis in body tissues. In this study, it has been determined that JPGS can effectively reduce apoptosis in the kidney tissues of DN mice. JNK and P38-MAPK family members play a central role in inducing cell apoptosis. JNK, a type of serine-threonine kinase, can be activated by multiple factors (*e.g.*, stress and inflammatory cytokines). Activated JNK is translocated from the cytoplasm to the nucleus to further induce the mRNA transcription of pro-apoptotic factors (*e.g.*, p53, Fas ligand, TNF, and Bax) and to act on mitochondria. For example, Bax and Bak promote the production of cytochrome C in cytoplasm so that cytochrome C binds to caspase-9 to act on caspase-3. Activated caspase-3 binds to apoptotic substrates to cause cell apoptosis[71]. JNK and P38 are stress-activated protein kinases and some factors can activate both JNK and P38 pathways. Activated P38 can induce cell apoptosis by increasing c-myc expression, phosphorylating P53, inducing Bax translocation, and enhancing TNF-α expression[71]. As oxidative stress occurs and increased levels of inflammatory factors are present in DN patients, the JNK/P38 signaling pathway is activated to exacerbate kidney cell apoptosis[72]. As such, kidney injury in DN patients can be improved by inhibiting JNK/P38-mediated apoptosis[73].

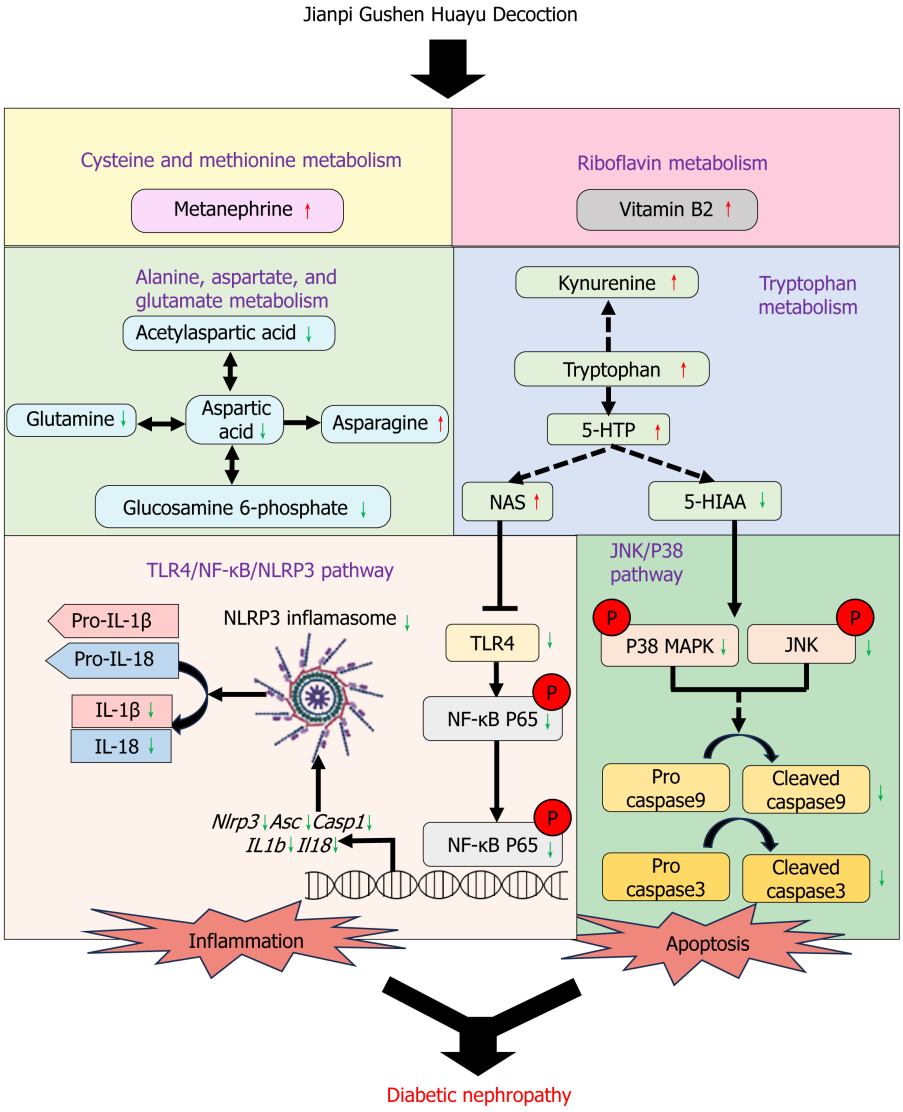

Figure 7 Jianpi Gushen Huayu Decoction could improve kidney inflammatory responses and kidney injury in diabetic nephropathy mice *via* the toll-like receptor 4/nuclear factor-kappa B/NOD-like receptor family pyrin domain containing 3 pathway and inhibit kidney cell apoptosis in diabetic nephropathy mice *via* the JNK/P38 pathway. It is possibly related to regulate cysteine and methionine metabolism, alanine, aspartate, and glutamate metabolism, tryptophan metabolism and riboflavin metabolism in kidney. NF-κB: Nuclear factor-kappa B; 5-HTTP: 5-hydroxytryptophan; NAS: N-acetylserotonin; 5-HIAA: 5-hydroxyindole-3-acetic acid; JNK: c-Jun N-terminal kinase; TLR4: toll-like receptor 4; NLRP3: NOD-like receptor family pyrin domain containing 3; ASC: Protein apoptosis-associated speck-like protein containing a CARD; IL: Interleukin.

CONCLUSION

Our results revealed that JPGS could markedly treat mice with STZ-induced DN. The metabolomics results exhibited that the efficacy of JPGS is possibly related to cysteine and methionine metabolism; alanine, aspartate, and glutamate metabolism; tryptophan metabolism; and riboflavin metabolism. Furthermore, JPGS could improve kidney inflammatory responses and kidney injury in DN mice *via* the TLR4/NF-κB/NLRP3 pathway and may inhibit kidney cell apoptosis in DN mice *via* the JNK/P38 pathway (Figure 7).

ARTICLE HIGHLIGHTS

Research background

As a frequent complication of type 2 diabetes mellitus, diabetic nephropathy (DN) is insidious and highly prevalent. The dysfunction of metabolism is closely related to the progression of diabetes and diabetic complications. Regulating metabolism to ameliorate DN has recently become a well explored research area.

Research motivation

Jianpi Gushen Huayu Decoction (JPGS) is highly efficacious clinically on DN, nevertheless, its renoprotective mechanism of action on DN has not yet been intensively investigated.

Research objectives

To evaluate the therapeutic effects and the possible mechanism of JPGS on DN based on untargeted metabolomics.

Research methods

The therapeutic potentials of JPGS was first evaluated on a DN mouse model. Then, the effect of JPGS on the kidney metabolite levels in DN mice was tested using non-targeted metabolomics. Furthermore, the effects of JPGS on c-Jun N-terminal kinase (JNK)/P38-mediated apoptosis and inflammatory responses mediated by toll-like receptor 4 (TLR4)/nuclear factor-kappaB (NF-κB)/NOD-like receptor family pyrin domain containing 3 (NLRP3) were examined.

Research results

JPGS administration improved body weight loss, hyperglycemia, kidney injury, and factors of oxidative stress and inflammation in DN mice. In addition, JPGS altered 51 differential metabolites of kidney in DN mice. Pathways related to cysteine and methionine metabolism, alanine, tryptophan metabolism, aspartate and glutamate metabolism, and riboflavin metabolism were identified as the key pathway of JPGS on DN. Further experimental validation showed that JPGS treatment reduced the expression of expression of TLR4/NF-κB/NLRP3 pathway and JNK/P38 pathway-mediated apoptosis related factors.

Research conclusions

JPGS could improve kidney inflammatory responses and kidney injury in DN mice *via* the TLR4/NF-κB/NLRP3 pathway and inhibit kidney cell apoptosis in DN mice *via* the JNK/P38 pathway. It is possibly related to regulate cysteine and methionine metabolism, alanine, aspartate, and glutamate metabolism, tryptophan metabolism and riboflavin metabolism in kidney.

Research perspectives

This study can provide scientific evidence for the clinal used of JPGS. Moreover, our study can be a useful for researchers in studying the relationship between metabolites and pathways in drug intervention.

FOOTNOTES

Author contributions: Cui HT and Lv SQ conceived and designed the study; Ma ZA, Wang LX, Zhang H, and Li HZ conducted the experiments and obtained the data; Li HZ, Dong L, and Wang QH analyzed and collated the data; Ma ZA, Wang YS, and Pan BC drafted and written the final version of the manuscript; Zhang SF, Cui HT, and Lv SQ contributed equally to this work; and all authors approved the final version of the manuscript.

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

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Basic Study

Diabetes and high-glucose could upregulate the expression of receptor for activated C kinase 1 in retina

Jian Tan, Ang Xiao, Lin Yang, Yu-Lin Tao, Yi Shao, Qiong Zhou

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Abstract

BACKGROUND

Diabetic retinopathy (DR) is a major ocular complication of diabetes mellitus, leading to visual impairment. Retinal pigment epithelium (RPE) injury is a key component of the outer blood retinal barrier, and its damage is an important indicator of DR. Receptor for activated C kinase 1 (RACK1) activates protein kinase C-ε (PKC-ε) to promote the generation of reactive oxygen species (ROS) in RPE cells, leading to apoptosis. Therefore, we hypothesize that the activation of RACK1 under hypoxic/high-glucose conditions may promote RPE cell apoptosis by modulating PKC-ε/ROS, thereby disrupting the barrier effect of the outer blood retinal barrier and contributing to the progression of DR.

AIM

To investigate the role and associated underlying mechanisms of RACK1 in the development of early DR.

METHODS

In this study, Sprague-Dawley rats and adult RPE cell line-19 (ARPE-19) cells were used as *in vivo* and *in vitro* models, respectively, to explore the role of RACK1 in mediating PKC-ε in early DR. Furthermore, the impact of RACK1 on apoptosis and barrier function of RPE cells was also investigated in the former model.

RESULTS

Streptozotocin-induced diabetic rats showed increased apoptosis and up-regulated expression of RACK1 and PKC-ε proteins in RPE cells following a prolonged modeling. Similarly, ARPE-19 cells exposed to high glucose and hypoxia displayed elevated mRNA and protein levels of RACK1 and PKC-ε, accompanied by an increases in ROS production, apoptosis rate, and monolayer permeability. However, silencing RACK1 significantly downregulated the

expression of PKC-ε and ROS, reduced cell apoptosis and permeability, and protected barrier function.

CONCLUSION
RACK1 plays a significant role in the development of early DR and might serve as a potential therapeutic target for DR by regulating RPE apoptosis and barrier function.

Key Words: Diabetic retinopathy; Receptor for activated C kinase 1; Protein kinase C-ε; Adult retinal pigment epithelium cell line-19

Core Tip: To investigate the role and associated underlying mechanisms of receptor for activated C kinase 1 (RACK1) in the development of early diabetic retinopathy (DR). In this study, Sprague-Dawley rats and adult retinal pigment epithelium (RPE) cell line-19 cells were used as *in vivo* and *in vitro* models, respectively, to explore the role of RACK1 in mediating protein kinase C-ε in early DR. RACK1 plays a significant role in the development of early DR, and may serve as a potential therapeutic target for DR by regulating the apoptosis and barrier function of RPE cells.

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INTRODUCTION

Diabetic retinopathy (DR) is the main ocular complication of diabetes mellitus (DM) and a common cause of visual impairment and blindness. The International Diabetes Federation predicts that the number of DM patients worldwide will increase from 460 million in 2019 to 700 million in 2045[1], and approximately 30% of DM patients will eventually develop DR[2]. Without effective treatment, DR patients will experience visual impairment or even blindness; hence, precise treatment in the early stage of DR is particularly important[3]. Retinal pigment epithelium (RPE) is the main component of the outer blood-retinal barrier (oBRB), and leakage caused by blood-retinal barrier injury is a sign of DR[4]. However, to date, research on DR has mostly focused on the internal retinal barrier, while research on the damage mechanism of the oBRB in diabetes is limited.

In the early stage, through bioinformatics analysis of retinal tissue samples of DR patients and normal people without diabetes, our research team obtained four hub genes and found that only the receptor for activated C kinase 1 (RACK1) was the most highly and differentially expressed (P = 0.003) hub gene (P = 0.003)[5]. RACK1 is a multifunctional signal transduction protein, also known as the anchoring protein of protein kinase C (PKC)[6]. PKC is a serine/threonine kinase involved in signal transduction that can respond to the stimulation of specific hormones, neurons, and growth factors[7]. The PKC family consists of 12 subtypes, among which PKC-α, -β, -δ, and-ε are activated and play an important role in the occurrence and development of DR[7,8]. Among them, PKC-ε can enhance the activity of nicotinamide adenine dinucleotide phosphate oxidase, thus promoting the production of reactive oxygen species (ROS) in RPE cells. The overaccumulation of ROS induces mitochondrial damage, apoptosis, inflammation, lipid peroxidation, and structural and functional changes in the retina[9].

Based on the above-mentioned research, we hypothesized that the activation of RACK1 under hypoxic/high-glucose (HG) conditions might promote apoptosis and migration of RPE cells by modulating PKC-ε/ROS, thereby disrupting the oBRB and leading to the progression of early DR. Therefore, this study aims to investigate the impact of knockdown RACK1 on alleviating PKC-ε/ROS induced damage to the oBRB, thus delaying the progression of early DR. RPE cells are highly polarized monolayer cells that are usually induced by HG to simulate the DR environment[10]. Considering that HG concentration and hypoxia are the two main components in the diabetic environment, we used both hypoxia and HG concentration to simulate this environment[9].

MATERIALS AND METHODS

Animal model

All male Sprague-Dawley rats (8 wk old, weighing 180-220 g) were purchased from the Animal Center of Nanchang University. All rats were housed in standard rat cages under standardized environmental conditions with controlled temperature (23 ± 2 °C), humidity (50%), and a 12-h light/dark cycle. The diabetic group received intraperitoneal injection of streptozotocin (STZ) (60 mg/kg body weight, Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) dissolved in citrate buffer (pH 4.5), while the control group received an equivalent volume of citrate buffer. Rats were

considered diabetic if their blood glucose levels exceeded 16.7 mmol/L 72 h after STZ injection and remained elevated for 1 wk. A total of 24 rats were included in the study, with 12 rats in each group. The rats were raised for 8 or 10 wk (n = 6 per group). All experiments were conducted according to the guidelines for the care and use of laboratory animals and approved by the Medical Ethics Committee of the First Affiliated Hospital of Nanchang University.

Hematoxylin and eosin staining of the retina

Six rats from each group were sacrificed, and their eyeballs were harvested at 8 and 10 wk after successful modeling. The eyeballs were then fixed in a 20% paraformaldehyde solution at 4 ℃ for 2 h. Subsequently, the samples were sectioned into 5 μm slices, stained with hematoxylin and eosin, and examined under a light microscope (magnification, 400 ×; Zeiss, OberCoring, Germany), to determine the number of RPE cells in the samples.

Cells and culture

Adult RPE cell line-19 (ARPE-19) cells were purchased from Procell (Wuhan, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) (containing 5.5 mM glucose) (Procell, Wuhan, China) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, United States) and 1% penicillin/streptomycin (Thermo Fisher) in 5% CO_2 at 37 ℃. The HG and hypoxia cell model was chemically induced by the adjustment of the glucose concentration of the culture medium to 25 mM, while 400 μM cobalt chloride ($CoCl_2$) (Merck, Germany) was added to the cell culture medium for 24 h before the experiment.

Small interfering RNA transfection

The RACK1-specific small interfering RNA (siRNA) (5′-3′ *GTCTCTGGATCTCGAGATA*) used in this study was obtained from Ruibo RIBOBIO (China). Transfection was performed in ARPE-19 cells when they reached 50%-70% confluency. The transfection was carried out using Lipofectamine 2000 (Invitrogen) with 100 nmol of RACK1 siRNA, and the medium was replaced with fresh medium 4-6 h after transfection. Transfected ARPE-19 cells were subsequently cultured for 48 h for mRNA experiments or 72 h for cell function and protein expression experiments.

Flow cytometry

ARPE-19 cells were grown in DMEM supplemented with 10% FBS for 12 h in a six-well plate, followed by treatment with HG and hypoxia for 24 h. The treated cells were collected (1 × 10^5 cells/mL) after digestion with pancreatic enzyme (Solarbio, Beijing, China) without ethylenediaminetetraacetic acid and then washed twice with pre-cooled phosphate-buffered saline (PBS). The cells were resuspended in 100 μL of binding buffer and stained with 5 μL Annexin V-FITC and 10 μL propidium iodide (Yeasen, Shanghai, China) for 15 min while protected from light for 15 min. Subsequently, 400 μL of binding buffer was added to resuspend the cells. The percentage of apoptotic cells was analyzed by flow cytometry (BD, FACSCalibur, United States).

Real-time quantitative polymerase chain reaction

Total RNA was extracted at room temperature using TRIzol reagent (Invitrogen; Thermo Fisher Scientific) and immediately reverse transcribed or stored at -80 ℃ as needed. mRNA was reverse transcribed into cDNA using the Strand cDNA Synthesis SuperMix for quantitative polymerase chain reaction (qPCR) kit (11141ES60, Yeasen, China) and subsequently quantified using specific primers from the SANGON primer group (Shanghai, China) for each mRNA. Real-time qPCR (RT-qPCR) was performed using the ABI PRISM 145 real-time polymerase chain reaction system (Applied Biosystems; Thermo Fisher Scientific) that used the Quick Start General SYBR Green (Roche, Basel, Switzerland). The cycle threshold (Ct) values were obtained, and relative mRNA expression levels were calculated based on the $2^{-\Delta\Delta Ct}$ method. The oligonucleotide sequences of RT-qPCR primers are listed in Table 1.

Western blotting analysis

Crushed tissue or cells were lysed using a buffer (Thermo Fisher Scientific, Waltham, United States) to extract proteins. The protein extracts were subsequently separated using 10% SDS-PAGE, and the separated proteins were transferred onto a PVDF membrane (Amersham, Cytiva, Germany). The membrane was incubated with 10% skim milk powder for 2 h to ensure its proper sealing. The membrane was then incubated overnight at 4 °C with a primary antibody for GAPDH (1:1000, Abcam), along with RACK1 and PKC-ε, to detect the target protein. The following day, a secondary antibody (1:5000, Abcam) conjugated with horseradish peroxidase was incubated with the membrane at room temperature for 1.5 h. Finally, the membrane was treated with an ECL reagent (Amersham Pharmacia Biotech, Inc., United States), and the protein bands were visualized using ImageJ software.

Determination of intracellular ROS

ROS were measured by assessing the intracellular peroxide-dependent oxidation of 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) that produces the fluorescent compound 2′, 7′-dichlorofluorescein. Cells were seeded in 24-well plates at a density of 2 × 10^4 cells per well and cultured for 24 h. After two washes with PBS, the cells were treated with fresh medium containing 25 mM glucose and 400 μM $CoCl_2$ and incubated for an additional 24 h. Subsequently, 20 μM DCFH-DA was added, and the cells were incubated for 30 min at 37 °C. After two more washes with PBS, 400 μL of PBS was added to each well, and the fluorescence intensity was measured using a TECAN SPARK 450M (Tecan Group, Ltd., Manedoff, Switzerland).

Table 1 Primer sequences for quantitative reverse transcription-polymerase chain reaction

Genes	Forward primers (5'-3')	Reverse primers (5'-3')
RACK1	*AAGCTGAAGACCAACCACA*	*GTCCCCACCATCTAGCG*
PKC-ε	*AGCCTCGTTCACGGTTCTATGC*	*GCAGTGACCTTCTGCATCCAGA*
β-actin	*GAGCTACGAGCTGCCTGACG*	*CCTAGAAGCATTTGCGGTGG*

RACK1: Receptor for activated C kinase 1; PKC- ε: Protein kinase C-ε.

Monolayer permeability assay

A 0.4-μm pore polycarbonate membrane insert with a 6.5-mm Transwell assay format (3413, Corning, NY, United States) was used to assess the vascular permeability of ARPE-19 cells. In each well, 105 cells in 200 μL of complete medium were transferred to the top chamber, while the bottom chamber was filled with 500 μL of the same medium. To conduct the permeability assays, the top chamber was loaded with 100 μL of a 1 mg/mL solution of fluorescein isothiocyanate dextran (FITC-dextran) (40 kDa, FD40, Sigma-Aldrich, St. Louis, MO, United States), while the bottom chamber was filled with 500 μL of PBS. Following a 30-min incubation in the darkroom, 100 μL samples were collected from the bottom chamber and plated onto 96-well plates. The leakage of FITC-dextran was subsequently analyzed using a TECAN SPARK 450M (Tecan Group, Ltd., Manedoff, Switzerland), with an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

Statistical analysis

A minimum of three repetitions were conducted for each experiment. All data were presented as mean ± SD. Statistical analyses were performed using Prism 9.0 software (GraphPad Software, San Diego, CA, United States) with Student's *t*-test and one-way ANOVA. $P < 0.05$ indicated statistical significance.

RESULTS

STZ-induced rats exhibited a typical diabetic phenotype

In our study, an animal model of diabetes was established through STZ injection. Both rat body weight and blood glucose levels were measured at weeks 0, 2, 4, 6, 8, and 10 to assess the progression of diabetes (Figure 1). The results showed a significant increase in blood glucose levels in diabetic rats, which was > 4 times higher than those in the normal control group throughout the study. Additionally, diabetic rats experienced significant weight loss compared to the normal control group.

In this study, we examined the number of cells in the RPE layer in each group at various time points following STZ injection. Cell counting was performed at 0, 2, 4, 6, 8, and 10 wk post-injection (Figure 2). There was no significant difference in the number of RPE cells between the control and diabetic groups at 0, 2, 4, and 6 wk after STZ injection ($P > 0.01$). However, at weeks 8 and 10, a notable decrease in the number of RPE cells was observed in the diabetic group. Furthermore, a significant decrease in the number of RPE cells was observed in the diabetic group compared to the normal control group at week 8 and 10($P < 0.01$). These findings suggested a progressive loss of RPE cells in the diabetic group over time.

Aberrant expression of RACK1 and PKC- ε in the retina of diabetic rats

The protein levels of RACK1 and PKC-ε in retinal tissues of normal and diabetic rats were assessed using western blot analysis at weeks 8 and 10 following STZ injection (Figure 3). The findings revealed that the protein levels of RACK1 and PKC-ε were significantly elevated in the retinal tissues of diabetic rats as compared to the normal group ($P < 0.05$).

HG combined with hypoxia up-regulated transcription and increased protein levels of RACK1 and PKC-ε in ARPE-19 cells

ARPE-19 cells were subjected to HG hypoxic conditions to mimic diabetes *in vitro*. The results indicated that the transcription and protein levels of RACK1 and PKC-ε were significantly elevated in the HG hypoxia group (Hg + Hypo) compared to the control group ($P < 0.05$) (Figure 4).

Silencing of RACK1 in ARPE-19 cells under HG hypoxia down-regulated PKC-ε

We examined the mRNA and protein levels of PKC-ε by siRNA after siRNA-silencing of RACK1 expression in ARPE-19 cells exposed to HG hypoxic conditions (Figure 5). The results showed that the inhibition of RACK1 expression in ARPE-19 cells reduced PKC-ε transcription and protein levels under HG hypoxia ($P < 0.05$).

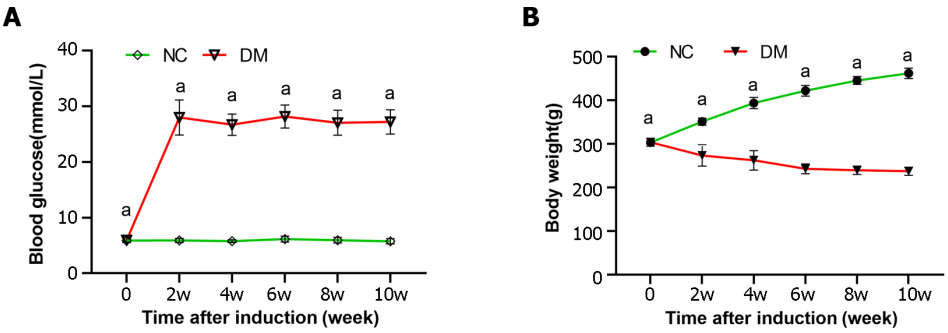

Figure 1 Blood glucose levels and body weight of rats following intraperitoneal injection of streptozotocin (65 mg/kg) (pH 4.5) in citrate buffer. A: Blood glucose level; B: Body weight. The results are presented as the mean ± SD. $^{a}P < 0.05$ *vs* control group ($n = 6$ in each group). NC: Normal control; DM: Diabetes mellitus.

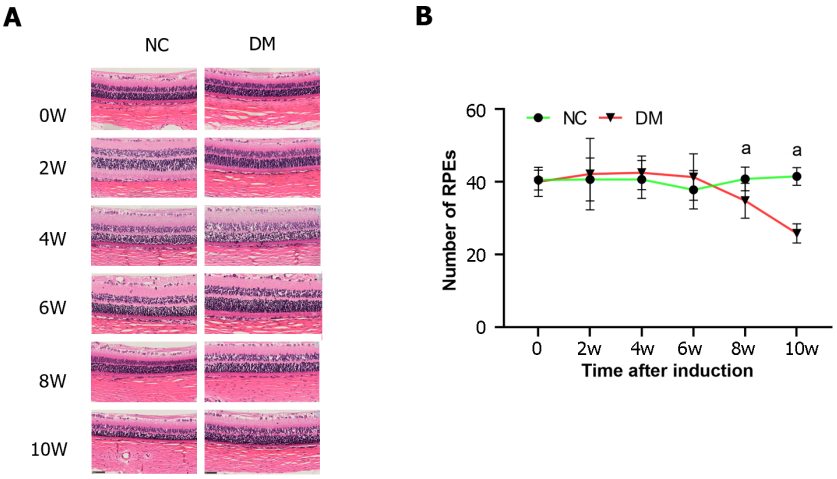

Figure 2 Retinal hematoxylin and eosin staining (magnification, 400) and retinal pigment epithelium cell counts were performed in normal and diabetic rats. A: The hematoxylin and eosin staining of rat retina; B: The quantification of retinal pigment epithelium cells. The results are expressed as the mean ± SD. $^{a}P < 0.05$ *vs* control group. NC: Normal control; DM: Diabetes mellitus.

Silencing RACK1 inhibits ROS elevation, apoptosis, and cell leakage in ARPE-19 cell monolayers under HG hypoxia

We subsequently examined ROS levels, apoptosis, and permeability between monolayers after silencing RACK1 expression by siRNA in ARPE-19 cells under HG hypoxia (Figure 6). The results showed that the inhibition of RACK1 expression in ARPE-19 cells down-regulated ROS levels, apoptosis, and cell permeability in monolayers under HG hypoxia ($P < 0.05$).

DISCUSSION

DR is a major ocular complication of diabetes that significantly impacts global health[11]. The mechanisms underlying its occurrence and development are complex and poorly understood[12]. Further elucidation of these mechanisms might aid in mitigating DR progression. RPE cells treated with HG are commonly used as an ideal model for investigating DR[13], and RPE cells are often exposed to HG and hypoxic conditions during DR development[14]. Studies suggested that the disruption of RPE barrier function in DR might result from apoptosis of RPE cells under HG and hypoxic conditions[15], although the specific mechanisms remain unclear. In this study, we aimed to explore the effect of RACK1 on RPE barrier function through both *in vitro* and *in vivo* experimental models to verify its role in the occurrence and development of early DR.

The RPE consists of highly specialized single-layer chromatophores, which are located between microvessels of the villus and outer segments of photoreceptors[16,17]. The RPE and photoreceptors in the outer retinal layer usually act as units to maintain normal visual function. Similarly, mutations in photoreceptor cells or RPE can lead to retinal degeneration[18]. In this study, we observed that apoptosis of RPE cells occurred in the retina of STZ-induced diabetic rats at week 8, highlighting apoptosis as an important factor in RPE and oBRB damage in early DR. Furthermore, we investigated the expression of RACK1 and PKC-ε in the retina of diabetic rats at weeks 8 and 10, showing that both mRNA and protein levels of RACK1 and PKC-ε were significantly higher in the retina of diabetic rats compared to the

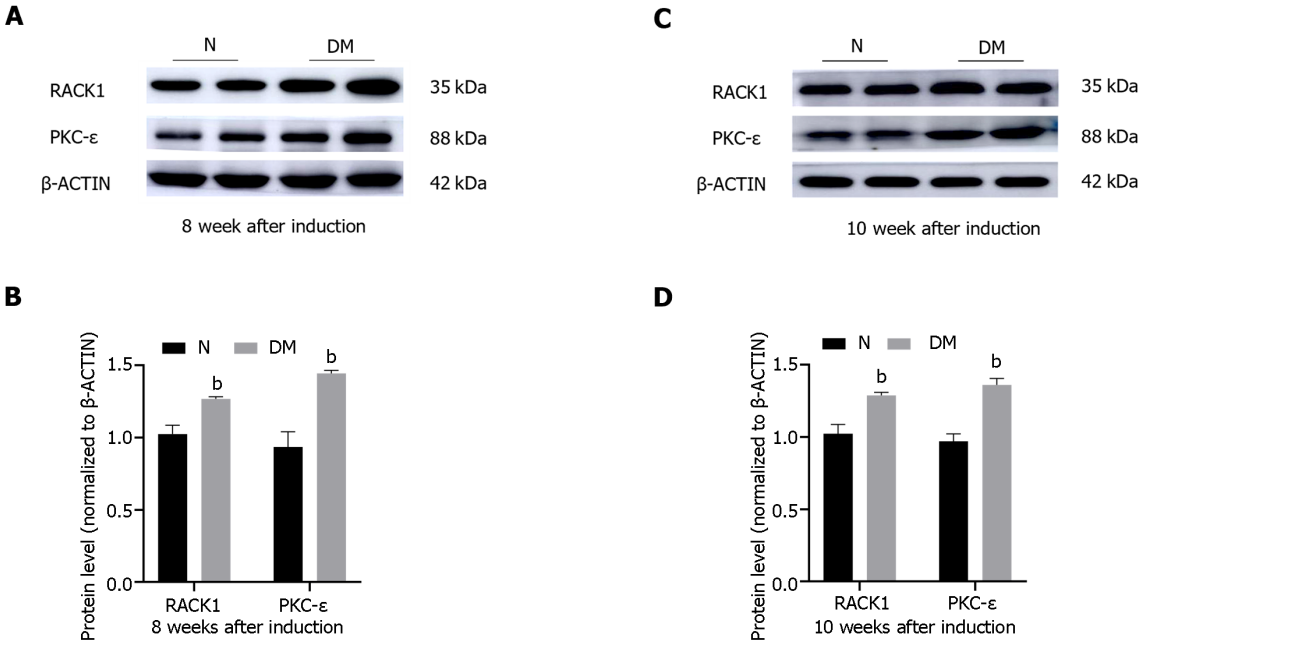

Figure 3 Aberrant expression of receptor for activated C kinase 1 and protein kinase C-ε in the retina of diabetic rats. A and B: The protein levels of receptor for activated C kinase 1 and protein kinase C-ε in the retinas of N and DM rats were measured at 8 wk following streptozotocin injection; C and D: The protein levels of receptor for activated C kinase 1 and protein kinase C-ε in the retinas of N and DM rats were measured at 10 wk following streptozotocin injection. The results are expressed as the mean ± SD. $^{b}P < 0.01$ *vs* control group. N: Normal control; DM: Diabetes mellitus; 8 W: 8 wk; 10W: 10 wk; RACK1: Receptor for activated C kinase 1; PKC- ε: Protein kinase C-ε.

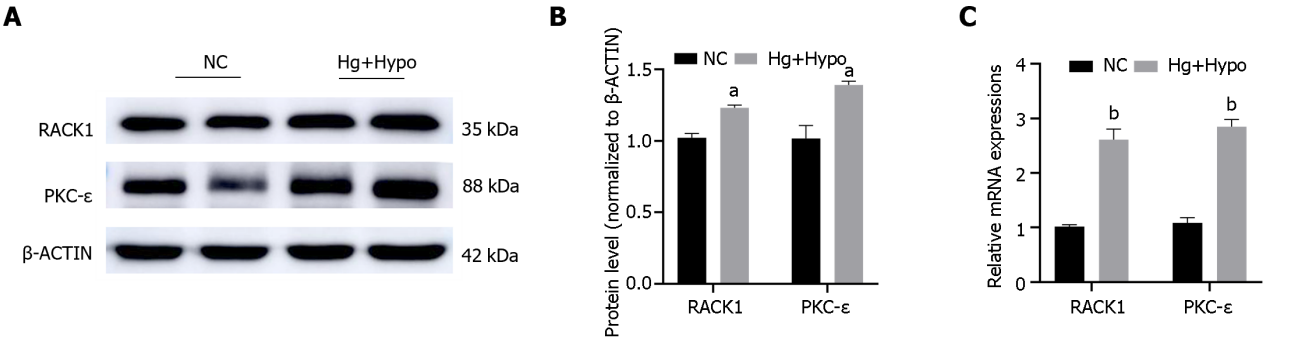

Figure 4 High glucose combined with hypoxia up-regulated transcription and increased protein levels of receptor for activated C kinase 1 and protein kinase C-ε in adult retinal pigment epithelium cell line-19 cells. A and B: The protein levels of receptor for activated C kinase 1 (RACK1) and protein kinase C-ε (PKC- ε) in the adult retinal pigment epithelium cell line-19 (ARPE-19) cells of normal control and high glucose combined with hypoxia; C: The ratio of mRNA of RACK1 and PKC- ε in ARPE-19 cells. The results are expressed as the mean ± SD. $^{a}P < 0.05$ *vs* control group. $^{b}P < 0.01$ *vs* control group. NC: Normal control; Hg + Hypo: High glucose combined with hypoxia; RACK1: Receptor for activated C kinase 1; PKC- ε: Protein kinase C-ε.

control group. Prior studies similarly demonstrated that elevated levels of RACK1 can promote cell apoptosis induced by polyglutamine[19], while the inhibition of PKC-ε can protect RPE cells from lipopolysaccharide-induced injury. However, whether PKC-ε can be suppressed by regulating RACK1 remains unclear[20]. In contrast, inhibition of RACK1 might potentially reduce damage and cell apoptosis in RPE cells in diabetes. Further investigations are needed to fully understand the mechanisms by which RACK1 and PKC-ε contribute to RPE cell damage and apoptosis in diabetes.

Therefore, in this study, we conducted an *in vitro* experiment to simulate the HG hypoxic environment of ARPE-19 cells in diabetes. We observed that mRNA and protein levels of RACK1 and PKC-ε in ARPE-19 cells were significantly higher under those conditions compared to the control group. RACK1 serves as a scaffold protein that mediates PKC activation[21]. PKC is a member of the family of serine/threonine protein kinases that are crucial in regulating many biological processes, such as cell division, growth, apoptosis, and cellular responses to environmental stressors. Meanwhile, the PKC pathway is an important pathway involved in DR.

However, mRNA and protein levels of PKC-ε were significantly reduced in the control group after inhibiting RACK1 expression, suggesting the possibility of down-regulating PKC-ε by inhibiting RACK1 expression. Hyperglycemia and tissue hypoxia in diabetes patients both increase the production of ROS, leading to retinal and tissue damage[22]. Our findings showed that the production of ROS by ARPE-19 cells was significantly increased under hyperglycemic and hypoxic conditions but could be significantly reduced by inhibiting RACK1. PKC is known to be involved in ROS

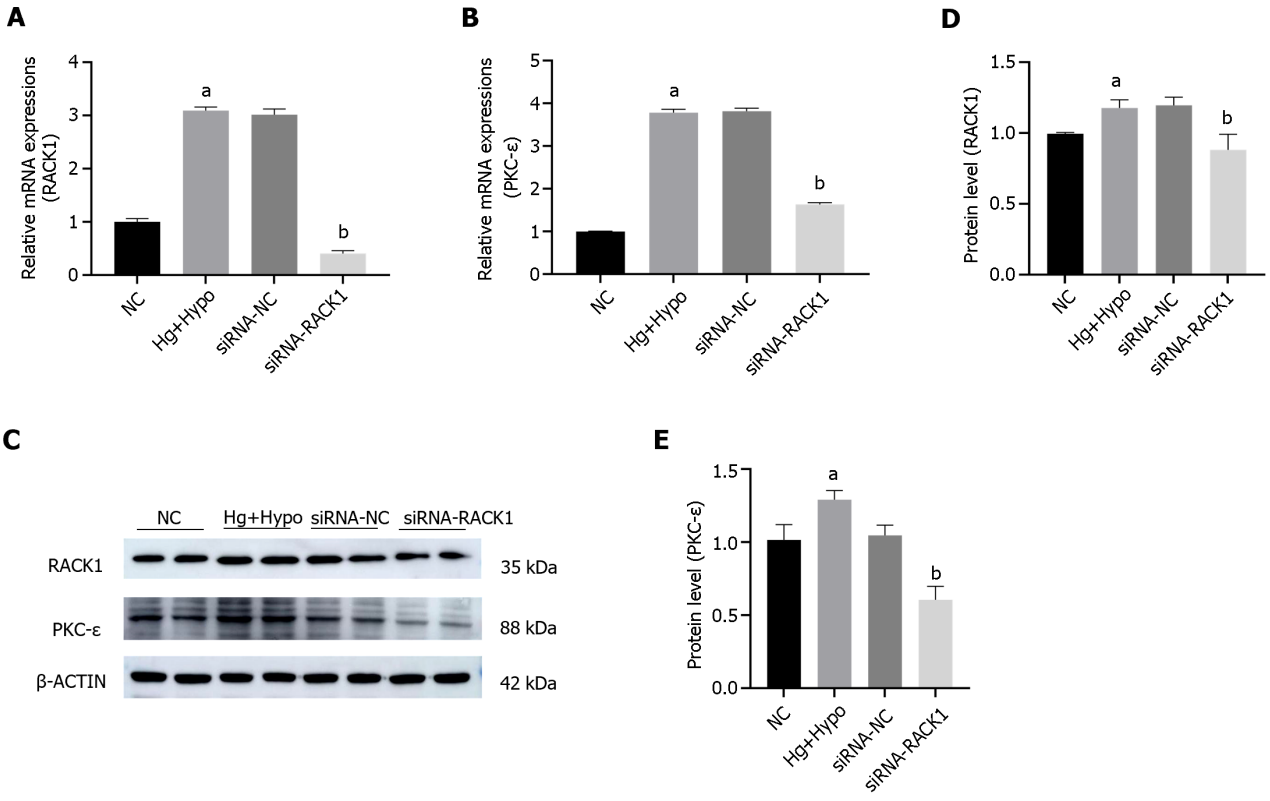

Figure 5 Silencing of receptor for activated C kinase 1 in adult retinal pigment epithelium cell line-19 cells under high glucose combined with hypoxia down-regulated protein kinase C-ε. A and B: The mRNA levels of receptor for activated C kinase 1 (RACK1) and protein kinase C-ε (PKC- ε) in the normal control, high glucose combined with hypoxia, non-silent siRNA, and silenced RACK1 siRNA groups; C-E: The protein levels of RACK1 and PKC-ε in adult retinal pigment epithelium cell line-19 cells of each group. The results are expressed as the mean ± SD. $^{a}P < 0.05$ *vs* normal control. $^{b}P < 0.05$ *vs* non-silent siRNA-normal control. NC: Normal control; Hg + Hypo: High glucose combined with hypoxia; siRNA-NC: Non-silent siRNA; siRNA-RACK1: Silenced RACK1 siRNA; RACK1: Receptor for activated C kinase 1; PKC- ε: Protein kinase C-ε.

production, and the increase in PKC stimulates ROS production in the mammalian target of rapamycin complex 1 pathway, which is related to autophagy[23]. PKC-ε plays a tissue-specific role in redox biology, with specific isoforms being both a target of ROS and an upstream regulator of ROS production[24]. Therefore, this effect might result in the accumulation of unfolded proteins and dysfunctional organelles in cells, contributing to DR pathophysiology[25]. Furthermore, our study also revealed that under HG and hypoxic conditions, ARPE-19 cell viability decreased, and apoptosis increased. This could potentially be attributed to the activation of PKC-ε under HG and hypoxic conditions, leading to increased ROS production and subsequent cell autophagy. Therefore, reduction of PKC-ε activation by inhibiting RACK1 expression might be possible, thereby decreasing ROS production and alleviating cell autophagy and cellular damage.

In diabetes, the activation of PKC is mediated by the formation of diacylglycerol (DAG), a physiological activator of PKC[26]. Blocking DAG, the activator of PKC, can interrupt the metabolic signaling cascade and inhibit ROS production. Therefore, inhibiting DAG formation is a potential method to control this signaling pathway[27]. During the onset and progression of diabetes, the inhibition of phospholipase D and phospholipase C can lead to an increase in the level of DAG through de novo synthesis. This increase in DAG levels can contribute to the induction of more severe oxidative stress in diabetes[28-30]. Phosphate hydrolase 1 and 2 can catalyze the conversion of phosphatidic acid into DAG through a process called de novo synthesis[31,32]. However, because of its biochemical complexity and multiple sources, direct DAG inhibition is not the best treatment option for diabetes. RACK1, as a scaffold protein involved in multiple signal transduction cascades, can promote the expression of PKC and enhance its activity in cells in a manner highly dependent on PKC-ε[33,34]. This makes it a promising therapeutic target to replace DAG inhibition. By inhibiting RACK1, the expression and activity of PKC-ε can be reduced, leading to a decrease in ROS production, potentially mitigating oxidative stress in diabetes. Furthermore, HG and hypoxia can induce the expression of apoptosis-promoting transcription factor C/EBP homologous protein in ARPE-19 cells and disrupt the integrity of tight junctions[35]. In our study, we observed that silencing RACK1 reduced FITC leakage in ARPE-19 cells under HG and hypoxia conditions. However, the specific downstream mechanisms related to the changes in tight junction proteins are not yet fully understood and require further research to elucidate the underlying mechanisms by which RACK1 disrupts the oBRB.

However, this study has certain limitations. First, all mechanistic experiments were conducted in ARPE-19 cells. Future studies need to confirm the effect of RACK1 on the oBRB in diabetic rats. Studies conducted solely in cell lines, such as ARPE-19 cells, might not fully reflect the in vivo effects of RACK1 on the oBRB in diabetic rats or other animal models. Therefore, animal models are essential for studying complex physiological processes and evaluating potential therapeutic

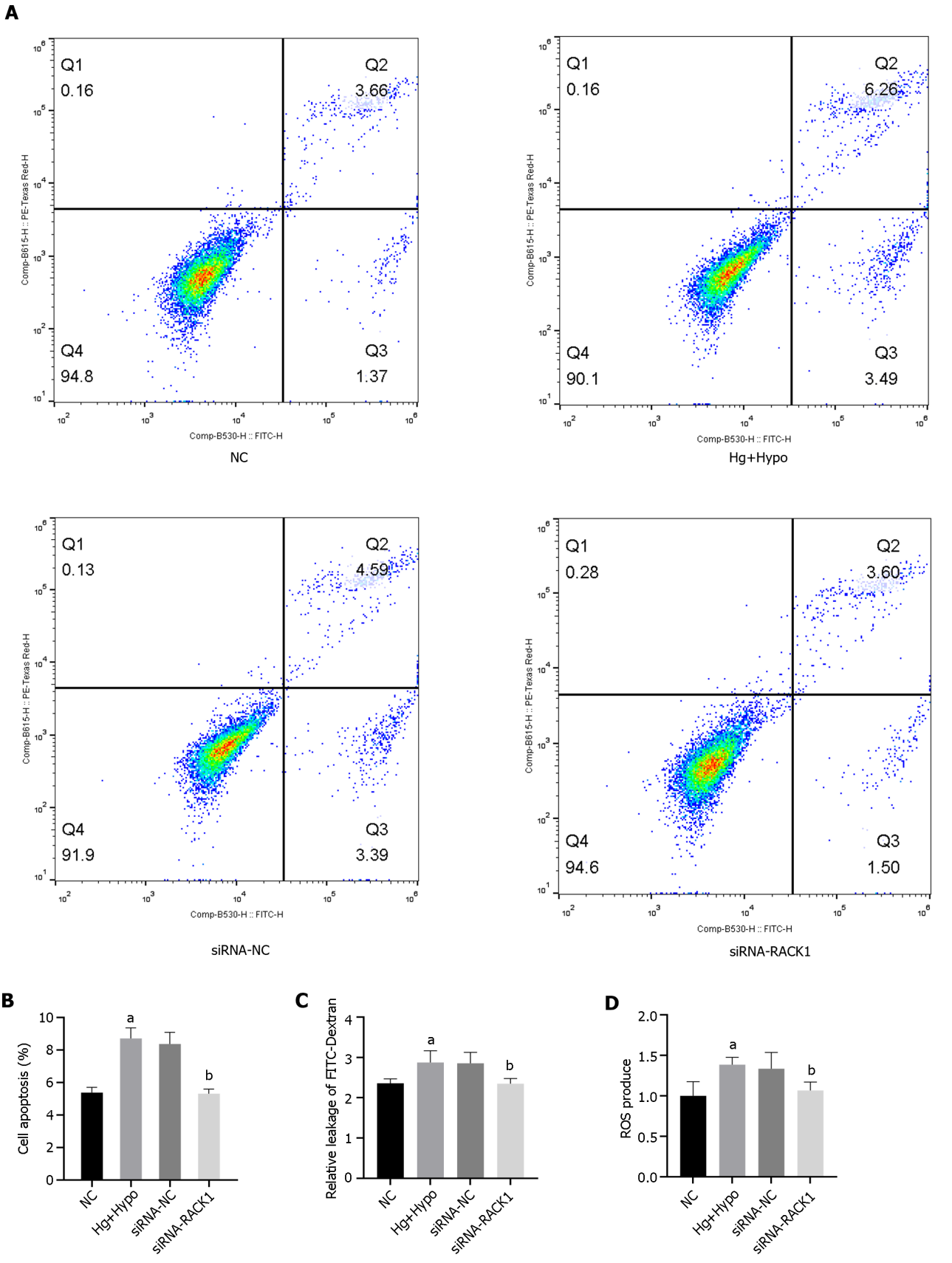

Figure 6 Silencing receptor for activated C kinase 1 inhibits reactive oxygen species elevation, apoptosis, and cell leakage in adult retinal pigment epithelium cell line-19 cell monolayers under high-glucose hypoxia. A and B: The apoptosis rate of adult retinal pigment epithelium cell line-19 cells was measured in the normal control (NC), high glucose combined with hypoxia, non-silent siRNA-NC, and silenced receptor for activated protein kinase C1 siRNA groups under high glucose hypoxia; C: The FITC-dextran leakage level in the cells of each group, used to analyze the permeability between cell levels in monolayers; D: The reactive oxygen species (ROS) production level of ROS detection kit in the cells of each group. The results are expressed as the mean ± SD. ^{a}P

< 0.05 *vs* normal control. [b]*P* < 0.05 *vs* siRNA-normal control. NC: Normal control; Hg + Hypo: High glucose combined with hypoxia; siRNA-NC: Non-silent siRNA; siRNA-RACK1: Silenced RACK1 siRNA; ROS: Reactive oxygen species; RACK1: Receptor for activated C kinase 1.

interventions because they provide a more comprehensive understanding of in vivo effects, including systemic factors and interactions between different cell types within the tissues of interest. Therefore, future studies should aim to confirm the effect of RACK1 on the oBRB in animal models of diabetes, such as diabetic rats. These studies might assess the expression and localization of RACK1, PKC isoforms, and DAG signaling components in the retina of diabetic animals. Additionally, functional assays can be performed to evaluate the integrity and permeability of the oBRB.

CONCLUSION

Knockdown of RACK1 can reduce PKC-ε activity and ROS production, thereby alleviating cellular oxidative stress and inflammatory responses. By reducing the excessive activation of PKC-ε/ROS, the occurrence and progression of early DR can be reduced. This may be achieved through the reduction of cellular oxidative stress and inflammatory response, improvement of retinal cell survival and function, and the reduction of vascular lesions and inflammatory infiltration. Therefore, inhibiting RACK1 might be a potential therapeutic strategy to slow down the progression of early DR by regulating PKC-ε/ROS. However, further research is needed to determine the safety and efficacy of this strategy and definitively explore its potential clinical applications.

ARTICLE HIGHLIGHTS

Research background

Diabetic retinopathy (DR) is a major ocular complication of diabetes mellitus, leading to visual impairment. Retinal pigment epithelium (RPE) injury is a key component of the outer blood retinal barrier, and its damage is an important indicator of DR.

Research motivation

Therefore, inhibiting receptor for activated C kinase 1 (RACK1) may be a potential therapeutic strategy to slow down the progression of early DR by regulating protein kinase C-ε/ reactive oxygen species (PKC-ε/ROS).

Research objectives

Knockdown of RACK1 can reduce the activity of PKC-ε and the production of ROS, thereby alleviating cellular oxidative stress and inflammatory responses. By reducing the excessive activation of PKC-ε/ROS, the occurrence and progression of early DR can be reduced.

Research methods

In this study, Sprague-Dawley rats and adult RPE cell line-19 (ARPE-19) cells were used as *in vivo* and *in vitro* models, respectively, to explore the role of RACK1 in mediating PKC-ε in early DR. Furthermore, the effect on the apoptosis and barrier function of RPE cells was also investigated in the former model.

Research results

Knockdown of RACK1 can reduce the activity of PKC-ε and the production of ROS, thereby alleviating cellular oxidative stress and inflammatory responses. By reducing the excessive activation of PKC-ε/ROS, the occurrence and progression of early DR can be reduced.

Research conclusions

this study proposes that by reducing the excessive activation of PKC-ε/ROS, the occurrence and progression of early DR can be reduced. This may be achieved through the reduction of cellular oxidative stress and inflammatory response, improvement of retinal cell survival and function, and the reduction of vascular lesions and inflammatory infiltration.

Research perspectives

One of the main limitations of this study is that all the mechanistic experiments were conducted in ARPE-19 cells. Future studies need to confirm the effect of RACK1 on the oBRB in diabetic rats.

FOOTNOTES

Co-first authors: Jian Tan and Ang Xiao.

Author contributions: Tan J, Xiao A, Yang L, Tao YL, Shao Y and Zhou Q designed the research study; Tan J, Xiao A and Yang L performed the research; Tan J and Shao Y contributed new reagents and analytic tools; Tan J and Zhou Q analyzed the data and wrote the manuscript; all authors have read and approve the final manuscript. Tan J and Xiao A contributed equally to this work as co-first authors. The reasons for designating Tan J and Xiao A as co-first authors are threefold. First, Tan J and Xiao A made equal contributions to the project research. Secondly, both Tan J and Xiao A actively participated in subsequent revisions and communication related to the paper. Finally, co-authorship serves to better exemplify collaboration within the team. In summary, we believe that designating Tan J and Xiao A as co-first authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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ORIGINAL ARTICLE

Basic Study

Potential application of *Nardostachyos Radix et Rhizoma*-Rhubarb for the treatment of diabetic kidney disease based on network pharmacology and cell culture experimental verification

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Abstract

BACKGROUND

Diabetic kidney disease (DKD) is one of the serious complications of diabetes mellitus, and the existing treatments cannot meet the needs of today's patients. Traditional Chinese medicine has been validated for its efficacy in DKD after many years of clinical application. However, the specific mechanism by which it works is still unclear. Elucidating the molecular mechanism of the *Nardostachyos Radix et Rhizoma*-rhubarb drug pair (NRDP) for the treatment of DKD will provide a new way of thinking for the research and development of new drugs.

AIM

To investigate the mechanism of the NRDP in DKD by network pharmacology combined with molecular docking, and then verify the initial findings by *in vitro* experiments.

METHODS
The Traditional Chinese Medicine Systems Pharmacology (TCMSP) database was used to screen active ingredient targets of NRDP. Targets for DKD were obtained based on the Genecards, OMIM, and TTD databases. The VENNY 2.1 database was used to obtain DKD and NRDP intersection targets and their Venn diagram, and Cytoscape 3.9.0 was used to build a "drug-component-target-disease" network. The String database was used to construct protein interaction networks. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis and Gene Ontology analysis were performed based on the DAVID database. After selecting the targets and the active ingredients, Autodock software was used to perform molecular docking. In experimental validation using renal tubular epithelial cells (TCMK-1), we used the Cell Counting Kit-8 assay to detect the effect of NRDP on cell viability, with glucose solution used to mimic a hyperglycemic environment. Flow cytometry was used to detect the cell cycle progression and apoptosis. Western blot was used to detect the protein expression of STAT3, p-STAT3, BAX, BCL-2, Caspase9, and Caspase3.

RESULTS
A total of 10 active ingredients and 85 targets with 111 disease-related signaling pathways were obtained for NRDP. Enrichment analysis of KEGG pathways was performed to determine advanced glycation end products (AGEs)-receptor for AGEs (RAGE) signaling as the core pathway. Molecular docking showed good binding between each active ingredient and its core targets. *In vitro* experiments showed that NRDP inhibited the viability of TCMK-1 cells, blocked cell cycle progression in the G0/G1 phase, and reduced apoptosis in a concentration-dependent manner. Based on the results of Western blot analysis, NRDP differentially downregulated p-STAT3, BAX, Caspase3, and Caspase9 protein levels ($P < 0.01$ or $P < 0.05$). In addition, BAX/BCL-2 and p-STAT3/STAT3 ratios were reduced, while BCL-2 and STAT3 protein expression was upregulated ($P < 0.01$).

CONCLUSION
NRDP may upregulate BCL-2 and STAT3 protein expression, and downregulate BAX, Caspase3, and Caspase9 protein expression, thus activating the AGE-RAGE signaling pathway, inhibiting the vitality of TCMK-1 cells, reducing their apoptosis. and arresting them in the G0/G1 phase to protect them from damage by high glucose.

Key Words: *Nardostachyos Radix et Rhizoma*-rhubarb; Diabetic kidney disease; Molecular docking; Network pharmacology; Experimental validation

Core Tip: The *Nardostachyos Radix et Rhizoma*-rhubarb drug pair may upregulate BCL-2 and STAT3 protein expression, and downregulate BAX, Caspase3, and Caspase9 protein expression, thus activating the advanced glycation end products (AGEs)-receptor for AGEs signaling pathway, inhibiting the vitality of TCMK-1 cells, reducing their apoptosis, and arresting them in the G0/G1 phase to protect them from damage by high glucose.

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INTRODUCTION

Diabetes mellitus (DM) is a clinical syndrome characterized mainly by elevated blood sugar caused by genetic factors. Delayed treatment will eventually lead to a series of serious complications, mainly diabetic kidney disease (DKD)[1]. DKD is one of the leading causes of end-stage renal disease, a leading cause of kidney failure. According to the epidemiological survey data released by the International Diabetes Federation, the global incidence of DM is 9.3%[2]; among them, 20%-40% develop DKD[3]. With the increase in the number of DKD patients, the treatment of DKD is imminent. The main treatment for DKD in Western medicine is to control blood sugar and improve renal function[4]. Clinical medications are mostly angiotensin-converting enzyme inhibitors and sulfonylureas to improve renal blood circulation. Despite this, the effect of these medications in relieving symptoms and reducing the disease's progression is not obvious. Hence, we desperately need to find effective drugs or compounds with minimal side effects to treat DKD[5].

As a traditional Chinese medicine in China, *Nardostachyos Radix et Rhizoma* belongs to the dried roots and rhizomes of *Nardostachys jatamansi*, a plant of the Septoria family[6]. Modern pharmacological studies have found that it is effective against brain diseases, heart diseases, spleen diseases, skin diseases, erectile dysfunction, tumors, and other diseases[7]. The chemical components of *Nardostachyos Radix et Rhizoma* are mainly terpenoids, coumarin, and lignans[8]. The active

compounds mansonopsin and naringin not only relieve cardiac hypertrophy[9] but also have anti-inflammatory, antibacterial, anti-osteoporosis, myocardium-protective, anti-malaria, liver-protective, anti-apoptosis, anti-tumor, sedative, antihypertensive, and anti-oxidative stress effects[10,11]. Other studies have shown that *Nardostachyos Radix et Rhizoma* can control blood glucose metabolism, regulate the islet function, and protect the kidney[12]. Rhubarb, which belongs to the dried roots and rhizome of *Rheum officinale Baill* in the Polygonum family, is widely utilized to cure diverse diseases. Rhubarb prevents the progression of DKD through a variety of mechanisms[13]. Modern pharmacological studies have found that anthraquinone derivatives contained in rhubarb have purgative effects[14]. Anthracene has an antidiarrheal effect. Emodin and rhein have anti-inflammatory, antibacterial, antiviral, anti-oxidative stress, anti-tumor, anti-fibrosis, lipid-regulating, and hypoglycemic effects[15,16]. Rhubarb tannin improves nitrogen waste metabolism; rhubarb anthraquinone and rhubarb anthraquinone glucoside can inhibit mesangial cell growth, improve renal tubular function, and protect the kidney[17]. Rhein has antitumor effects[18]. In addition, it has hemostatic, antiviral, antibacterial, liver-protecting[19], gallbladder-protecting, stomach-protecting[20], and kidney-protecting properties. In the treatment of DKD, rhubarb can reduce uremic toxin levels, regulate intestinal flora[14], and delay the progression of renal interstitial fibrosis. However, the drug targets and molecular mechanisms of *Nardostachyos Radix et Rhizoma*-rhubarb drug pair (NRDP) in the treatment of DKD have not been clarified. Therefore, we investigated the specific drug targets and molecular mechanisms of NRDP in the treatment of DKD based on network pharmacology combined with pharmacology.

DM and aberrant renal function are the causes of DKD. While the precise etiology remains unknown, certain research has demonstrated that advanced glycation end products (AGEs) formation is essential to the development of DKD. When the receptor for AGEs (RAGE) is activated, other associated pathways are impacted, which increases oxidative stress and inflammation in renal cells, encouraging apoptosis and exacerbating the progression of DKD[21]. Traditional Chinese medicine is often used to treat chronic diseases. Under high-glucose environment, the AGE-RAGE pathway will be activated to increase kidney damage. Studies have shown that traditional Chinese medicine monomers and compounds can regulate PI3K-AKT, NF-κB, JAK/STAT, and other pathways, and reduce oxidative stress, cell apoptosis, and inflammation, thereby improving kidney damage and delaying the course of DKD[22]. Since there have been few reports on the effects and mechanisms of NRDP in treating DKD, we analyzed the active components and targets of NRDP, and explored the mechanism underlying the therapeutic effects of NRDP on DKD in the present study.

Traditional Chinese medicine compounds exhibit multi-target, multi-component, and multi-pathway actions that are consistent with network pharmacology. In this study, we employed network pharmacology and experimental verification to confirm the mechanism of action of the compound on DKD. We searched for drug and disease targets using the TCMSP, Gene Cards, OMIM, and TTD databases, and then identified the core target pathway through the protein-protein interaction (PPI) network. Using NRDP components, we conducted Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses, as well as molecular docking. Finally, we performed experimental verification to prove the predictions made on the mechanism of NRDP in DKD (Figure 1), with an aim to provide new ideas and methods for the subsequent treatment of DKD with traditional Chinese medicine.

MATERIALS AND METHODS

Acquisition of active ingredients and targets of NRDP

The TCMSP database analysis platform (https://tcmsp-e.com/) was searched for the active ingredients and targets of *Nardostachyos Radix et Rhizoma* and rhubarb. The screening criteria for the active ingredients in drugs and their corresponding were oral availability ≥ 30% and drug likeness ≥ 0.18. Then, the UniProt (https://www.uniprot.org/) database was used to translate the targets into gene names.

Identification of DKD targets

In the GeneCards (http://www.genecards.org/), OMIM (http://omim.org/), and TTD (https://db.idrblab.net/ttd/) databases, "Diabetic Kidney Diseases" and "Diabetic Nephropathy" were searched as keywords, and the DKD targets were obtained.

Identification of intersection targets for NRDP and DKD

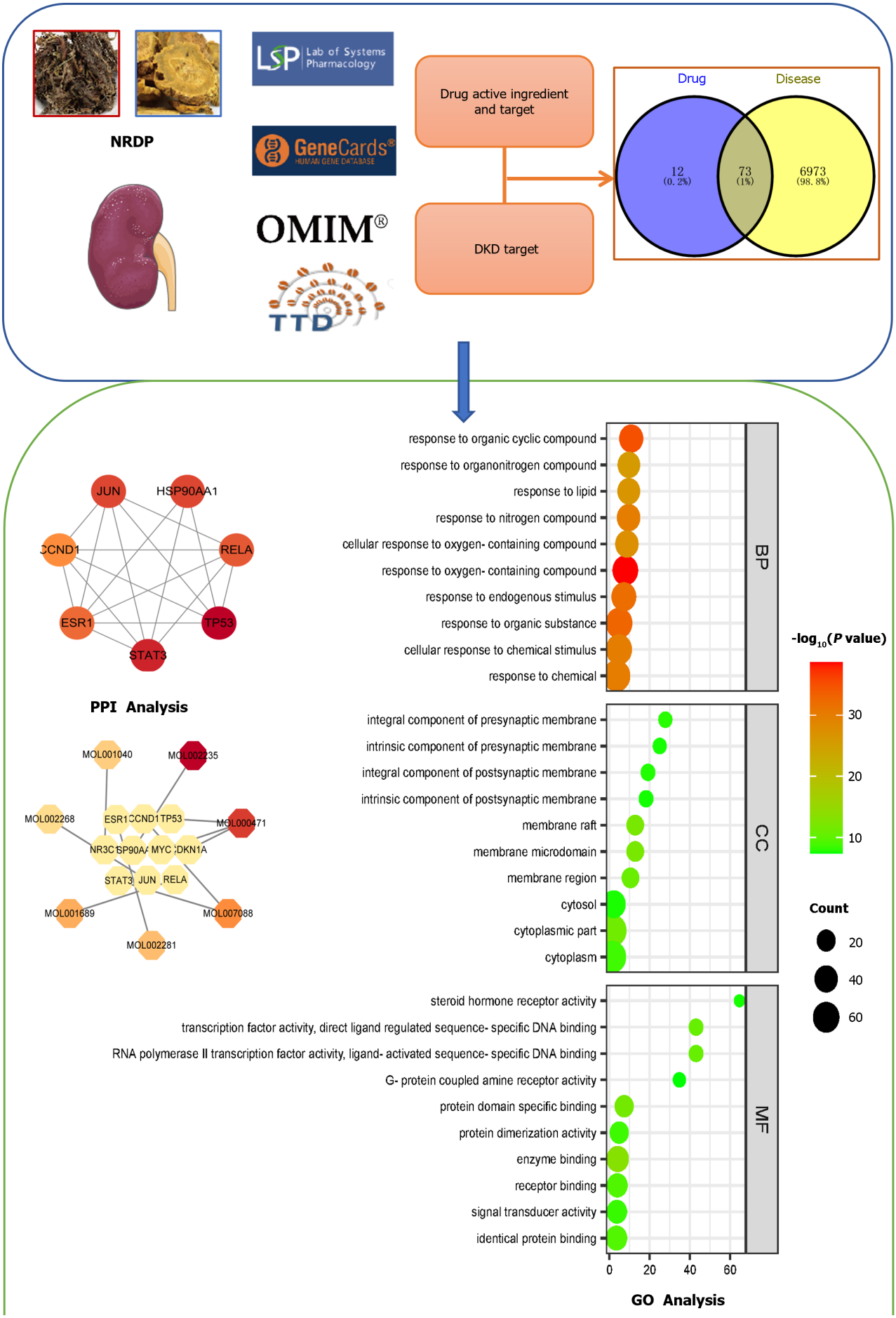
The Venny2.1.0 platform (https://bioinfogp.cnb.csic.es/tools/venny/) was used to obtain the common targets between the NRDP and DKD.

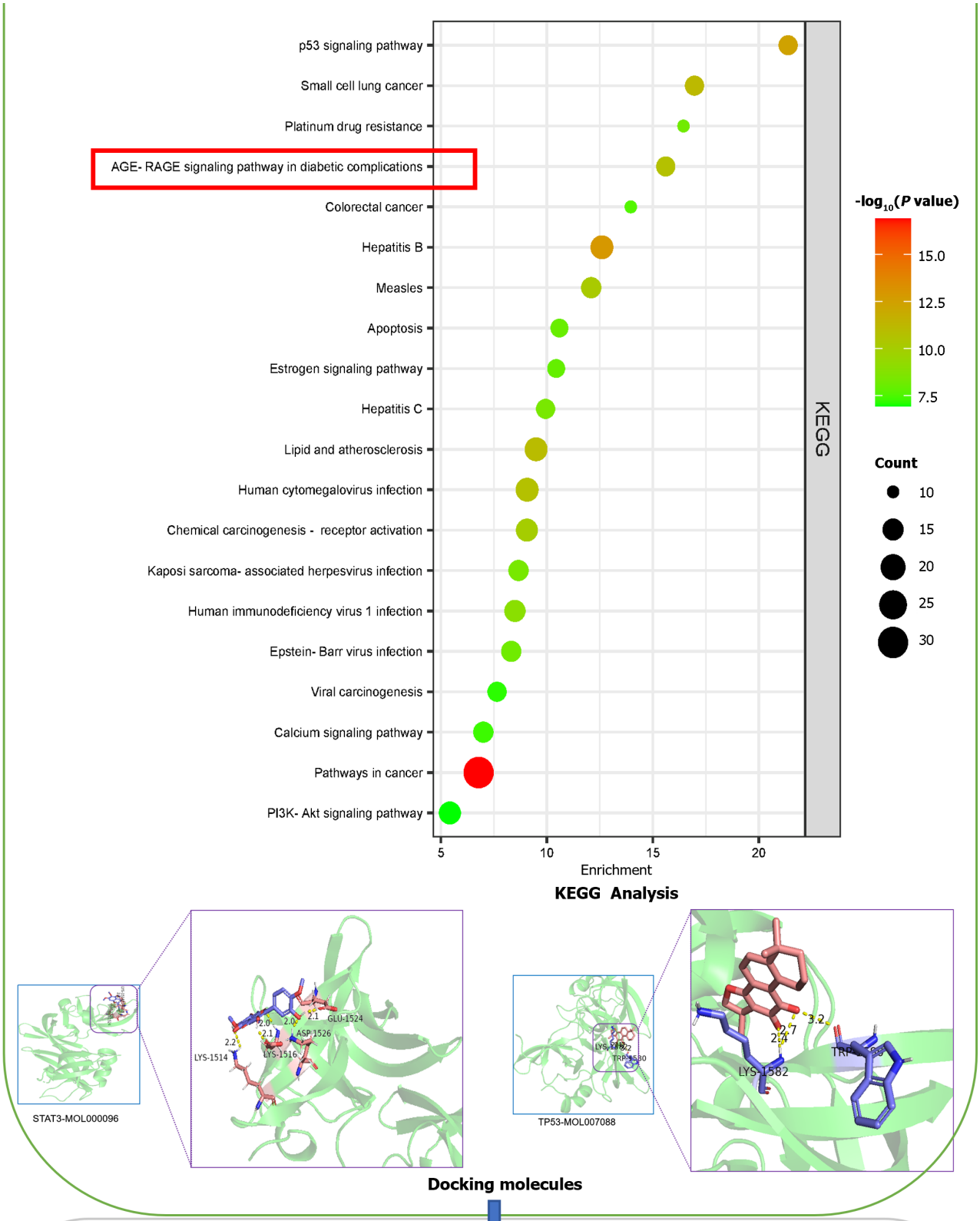
Construction of "component-target-disease" network

To visualize the results, a "component-target-disease" network was constructed using Cytoscape 3.9.0 with the active ingredients and targets of NRDP and DKD.

Construction of PPI network

PPI network diagrams were constructed by importing the common targets of NRDP and DKD into the STRING 11.5 database (https://cn.string-db.org/), and the species was set as "*Homo sapiens*". The minimum interaction threshold was set as "highest confidence" (> 0.9), to hide isolated nodes, and the rest of the settings were set as default. To obtain protein interaction data, the TSV file was downloaded and imported to Cytoscape software. The Network Analyzer plug-in was used to analyze network characteristics, and screen core targets in the PPI network according to the degree of nodes.


NRDP
Lab of Systems Pharmacology
GeneCards
HUMAN GENE DATABASE
OMIM®
TTD
Drug active ingredient and target
DKD target
Drug
Disease
12 (0.2%)
73 (1%)
6973 (98.8%)
JUN
HSP90AA1
CCND1
RELA
ESR1
TP53
STAT3
PPI Analysis
MOL001040
MOL002235
MOL002268
ESR1
CCND1
TP53
MOL000471
NR3C
HSP90AA
MYC
CDKN1A
STAT3
JUN
RELA
MOL001689
MOL007088
MOL002281
response to organic cyclic compound
response to organonitrogen compound
response to lipid
response to nitrogen compound
cellular response to oxygen- containing compound
response to oxygen- containing compound
response to endogenous stimulus
response to organic substance
cellular response to chemical stimulus
response to chemical
BP
integral component of presynaptic membrane
intrinsic component of presynaptic membrane
integral component of postsynaptic membrane
intrinsic component of postsynaptic membrane
membrane raft
membrane microdomain
membrane region
cytosol
cytoplasmic part
cytoplasm
CC
steroid hormone receptor activity
transcription factor activity, direct ligand regulated sequence- specific DNA binding
RNA polymerase II transcription factor activity, ligand- activated sequence- specific DNA binding
G- protein coupled amine receptor activity
protein domain specific binding
protein dimerization activity
enzyme binding
receptor binding
signal transducer activity
identical protein binding
MF
0
20
40
60
GO Analysis
-log10(P value)
30
20
10
Count
20
40
60


p53 signaling pathway
Small cell lung cancer
Platinum drug resistance
AGE- RAGE signaling pathway in diabetic complications
Colorectal cancer
Hepatitis B
Measles
Apoptosis
Estrogen signaling pathway
Hepatitis C
Lipid and atherosclerosis
Human cytomegalovirus infection
Chemical carcinogenesis - receptor activation
Kaposi sarcoma- associated herpesvirus infection
Human immunodeficiency virus 1 infection
Epstein- Barr virus infection
Viral carcinogenesis
Calcium signaling pathway
Pathways in cancer
PI3K- Akt signaling pathway
KEGG
5
10
15
20
Enrichment
-log10(P value)
15.0
12.5
10.0
7.5
Count
10
15
20
25
30
KEGG Analysis
STAT3-MOL000096
GLU-1524
ASP-1526
LYS-1514
LYS-1516
TP53-MOL007088
LYS-1582
3.2
2.7
2.4
Docking molecules
Experiment verification
120
90
60
50
30
0
Cell viability (%)
IC50=7.84(mg/ml)
0
5
10
15
Concentration (mg/ml)

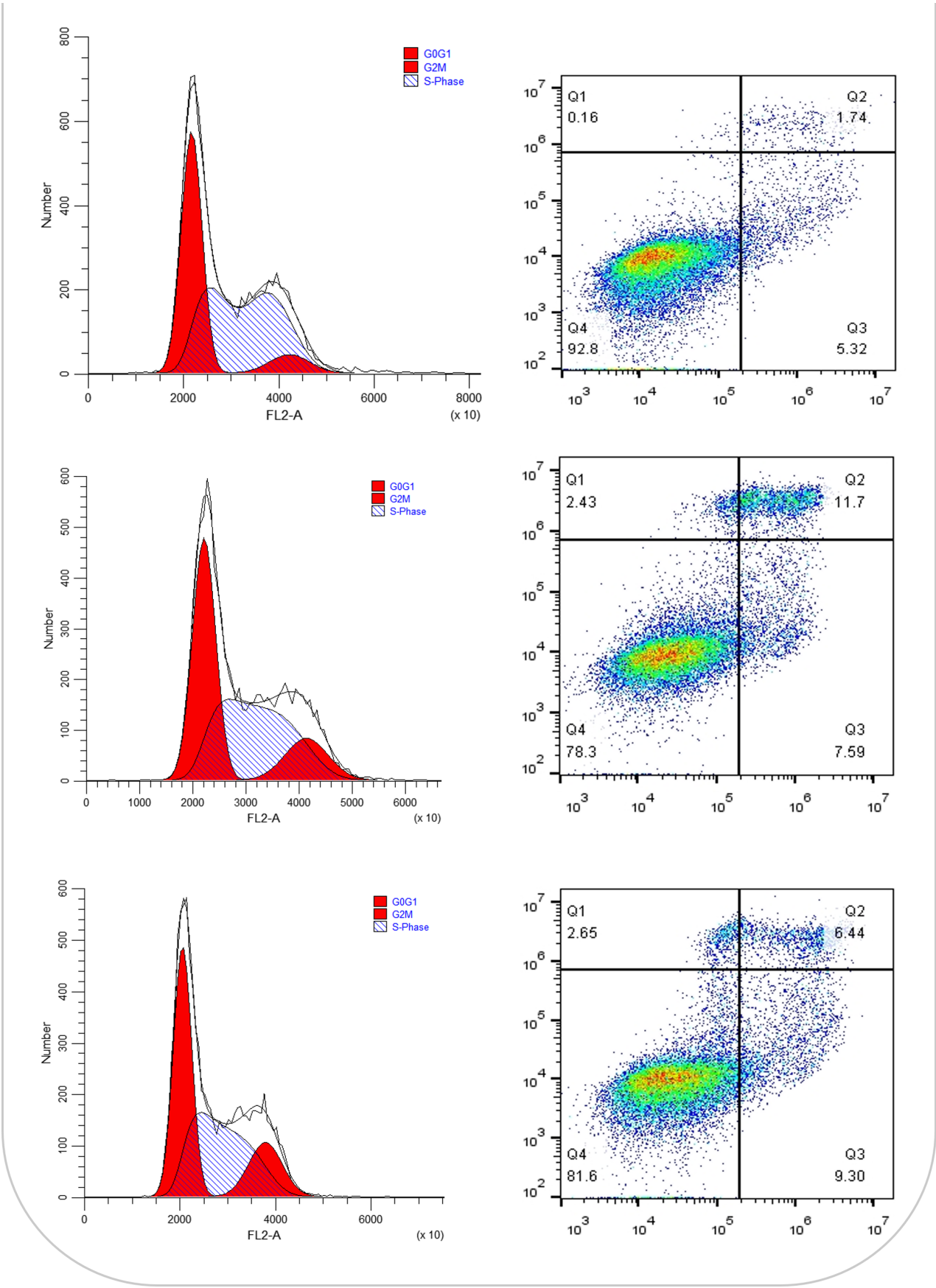


Figure 1 Flow chart of network pharmacological prediction and experimental validation. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DKD: Diabetic kidney disease; PPI: Protein-protein interaction; BP: Biological process; CC: Cell composition; MF: Molecular function; IC_{50}: Half maximal inhibitory concentration.

Composition-target network diagram construction

Cytoscape software was used to construct a network for common targets and NRDP active components. To analyze the characteristics of the network, the Network Analyzer plug-in was used, and the interaction between NRDP active components and core targets was analyzed according to the degree of nodes.

GO and KEGG enrichment analysis

Intersection targets were imported to the DAVID database (https://david.ncifcrf.gov/) for GO function and KEGG pathway enrichment analysis. *P* value < 0.01 and false discovery rate < 0.01 were used the conditions for the screening. With the help of online data analysis, the visualization platform - microscopic letter (http://www.bioin-formatics.com.cn/) resulted in visualization.

Construction of component-target network diagram on signaling pathway

The key pathway targets obtained following KEGG enrichment were used to generate a full PPI network based on the STRING11.5 database. The TSV file of PPI was downloaded and imported into Cytoscape software. After analysis with the Network Analyzer plug-in, a component-target network diagram about the pathway was reconstructed with the active components of NRDP. According to the degree of the node, the interaction between the active components of NRDP and the pathway targets was analyzed.

Molecular docking

NRDP medicine mol2 structures were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), and the core target protein 3D structure was downloaded from the PDB database (https://www.rcsb.org/). Then, the water molecules and small molecular ligands of proteins were removed using Pymol 2.4.0 software, and AutoDock 1.5.7 software was employed for hydrogenation. Molecular docking of receptor and ligand was performed and their binding activity was evaluated.

Cell experiment verification

Cells: TCMK-1 cells (renal tubule epithelial cells) were purchased from BeNa Culture Collection (No. BNCC339820).

Drugs and reagents: NRDP was prepared by the Preparation Center, Affiliated Hospital of Traditional Chinese Medicine, Ningxia Medical University.

Cell culture: TCMK-1 cells were cultured with complete medium (89% high-glucose DMEM + 10% fetal bovine serum + 1% penicillin-streptomycin mixture) in a 5% CO_2 incubator at 37 °C. A microscope was used to observe cell growth, and cells were passed at 80% confluence.

Determination of half inhibitory concentration of NRDP by CCK8 method

TCMK-1 cells in the logarithmic growth phase were digested with trypsin for cell suspension preparation. Cells were counted under a 20 times microscope, and 5 × 10^3 cells were inoculated per well into 96-well plates and incubated with complete cell culture medium (control group), 60 mmol/L high glucose culture medium (model group), or high glucose culture medium + different concentrations of NRDP (NRDP groups), with each group having five replicate wells. NRDP was diluted multiple times according to the drug concentration gradient, and then 100 μL of the diluted NRDP solution was added to each well and incubated for 24 h. At the end of the drug intervention, incubation with CCK8 (10 μL/well) was performed for 1 h under no light conditions. Optical density (OD) was then read at 450 nm.

Determination of effect of NRDP on the cell cycle of TCMK-1 cells treated with high glucose by flow cytometry

TCMK-1 cells in the logarithmic growth phase were divided into five groups: Control group (complete culture medium), model group (60 mmol/L high-glucose culture medium), low-dose group (high-glucose culture medium + 4 mg/mL NRDP), medium-dose group (high-glucose culture medium + 7 mg/mL NRDP), and high-dose group (high-glucose culture medium + 10 mg/mL NRDP). Three replicates were run for each group. Cells were inoculated into 6-well plates at a density of 1.0 × 10^5 cells/well. Following 24 h of culture in an incubator, serum-free medium was added to each group and incubated for 12 h. Cells were then digested, fixed overnight, and pre-cooled by adding 70% ethanol. A commercial cell cycle kit (KeyGEN Biotech, China) was used to detect the cell cycle progression with a CytoFLEX flow cytometer (Beckman Coulter, United States).

Detection of effect of NRDP on apoptosis of TCMK-1 cells treated with high glucose by flow cytometry

TCMK-1 cells in the logarithmic growth phase were divided into five groups as stated above, and three replicates were run for each group. Cells were inoculated into 6-well plates at a density of 1.0 × 10^5 cells/well. Following 24 h of culture in an incubator, each group of cells were exposed to the corresponding culture medium. After cells were digested and collected, the corresponding reagents were added according to the Annexin V-FITC/propidium iodide apoptosis detection kit (KeyGEN Biotech, China) instructions. The results were detected with a CytoFLEX flow cytometry (Beckman Coulter, United States).

Protein expression detection by Western blot analysis

TCMK-1 cells in the logarithmic growth phase were divided into three groups: Control group, model group, and medium dose group (7 mg/mL NRDP), and three replicates were run for each group. After cells were digested and collected, 200

μL of RIPA lysis buffer was added according to the total protein extraction kit (KeyGEN Biotech, China) instructions. The extracted proteins was subjected to protein content determination and then resolved by SDS-PAGE. After transfer to a membrane and membrane blockade, the membrane was incubated with the primary antibody at 4 °C overnight, followed by incubation with the secondary antibody for 1 h. Chemidoc (Ge1Doc XR+, BIO-RAD, United States) was used for chemiluminescence detection. Image J software was used to determine gray values for statistical analysis.

Statistical methods

GraphPad Prism 8.0.2 software was used for statistical analyses and one-way analysis of variance was used to compare the difference among groups. The SNK test was used to test for homogeneity of variances, and the Tamhane's *T* test was used to test for heterogeneity of variances. *P* values < 0.05 were regarded as statistically significant.

RESULTS

Chemical constituents and targets of NRDP

A total of 15 active ingredients were obtained from the TCMSP database, including five active ingredients from *Nardostachyos Radix et Rhizoma* and ten from rhubarb. After removing duplicate targets, 43 targets of *Nardostachyos Radix et Rhizoma*, 69 targets of rhubarb, and 85 GRDP targets were obtained (Figure 2A).

Drug-disease intersection targets and Venn diagram

Based on GeneCards (https://www.genecards.org/), OMIM (https://omim.org/), DrugBank (https://go.drug-bank.com/), and TTD databases (http://db.idrblab.net/ttd/), a total of 7046 relevant targets for DKD were screened (Figure 2A). A Venn diagram was drawn for 73 intersection targets between NRDP and DKD (Figure 2B).

Construction of drug-disease-active ingredient-target network

The NRDP active ingredients and common targets were imported into Cytoscape 3.9.0 software to obtain a visualized regulatory network diagram (Figure 2C). The nodes in the diagram include drug, disease, active ingredient, and target, where the edges indicate that there is an interrelationship between them. Orange represents diseases and drugs. Fuchsia represents the co-interacting active ingredients of NRDP-disease. Light blue represents the active ingredients of *Nardostachyos Radix et Rhizoma*. Light yellow represents the active ingredients of rhubarb. Light purple represents gene targets for NRDP-disease co-action. Pink represents the target genes for NRDP. Light orange represents target genes for rhubarb. This network diagram shows that NRDP works through multiple components and targets in the treatment of DKD.

Protein interaction network diagram

Using the STRING database, we obtained the protein interaction network diagram of NRDP and DKD (Figure 2D). There are 54 nodes and 310 edges, with the nodes representing proteins.

PPI network analysis

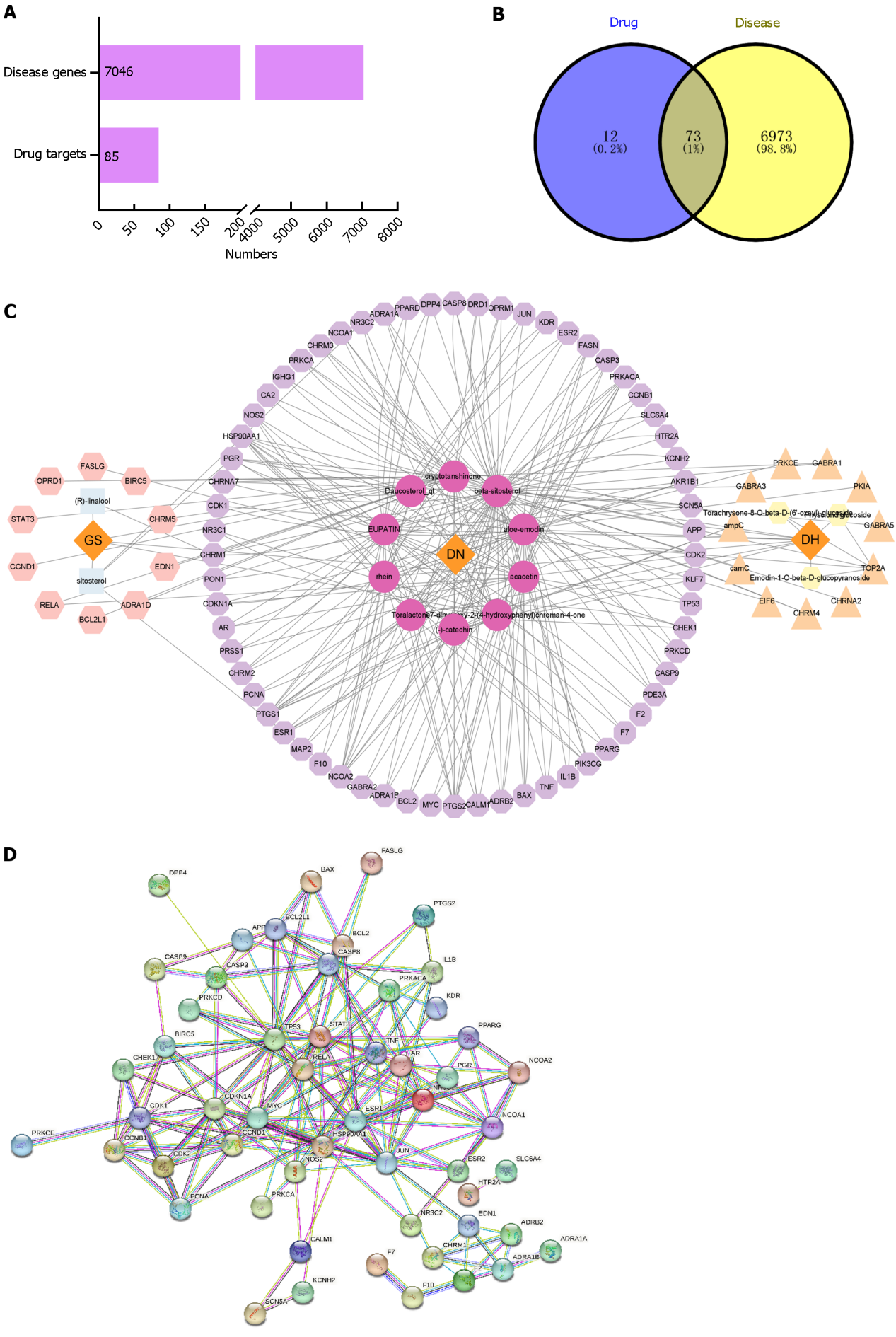
The TSV file of the above PPI network diagram was downloaded, and the Network Analyzer plug-in in Cytoscape was used to analyze the network characteristics, including 54 nodes and 178 edges. Nodes with a degree median greater than 13 were carded out to obtain the PPI network, including the core targets of NRDP for DKD treatment. The targets were TP53, STAT3, HSP90AA1, JUN, RELA, ESR1, CCND1, MYC, CDKN1A, NR3C1, and CDK1. The results are shown in Figure 2E.

Composition and target network analysis

A network diagram was constructed between PPI targets and NRDP active components. Through analysis with the Network Analyzer plug-in in Cytoscape software, the interaction between NRDP active components and 54 targets and their degree values were obtained. Nodes with a median of greater than 13 degrees were identified. Seven main components were obtained, including aloe-emodin, cryptotanshinone, EUPATIN, rhein, and acacetin, as shown in Figure 2F.

GO and KEGG analysis

A total of 1528 biological process entries were obtained after GO enrichment analysis, which mainly involves response to oxygen-containing compound and response to organic cyclic compound, cellular response to chemical stimulus, cellular response to oxygen-containing compound, *etc.* There were 107 cell composition items, including the membrane raft, cytoplasmic part, an integral component of the presynaptic membrane and plasma membrane, extracellular exosome, and cytosol. There were 115 molecular function entries, mainly involving enzyme binding, protein domain-specific binding, transcription factor activity, direct ligand regulated sequence-specific DNA binding, *etc.* The top 10 enrichment results of each group are plotted (Figure 3A). The KEGG pathway enrichment analysis identified 111 pathways. The top 20 pathways are plotted in Figure 3B. The important pathways involved in the therapeutic effects of NRDP on DKD include pathways in cancer, PI3K-AKT signaling pathway, p53 signaling pathway, AGE-RAGE signaling pathway in diabetic complications, and apoptosis signaling pathway. The AGE-RAGE signaling pathway is the core pathway. The component-target network diagram in the signal pathway was constructed with NRDP active components using the


A
Disease genes
7046
Drug targets
85
0
50
100
150
200
4000
5000
6000
7000
8000
Numbers
B
Drug
Disease
12
(0. 2%)
73
(1%)
6973
(98. 8%)
C
GS
DN
DH
(R)-linalool
sitosterol
cryptotanshinone
Daucosterol_qt
beta-sitosterol
EUPATIN
aloe-emodin
rhein
acacetin
(-)-catechin
Emodin-1-O-beta-D-glucopyranoside
ampC
camC
D

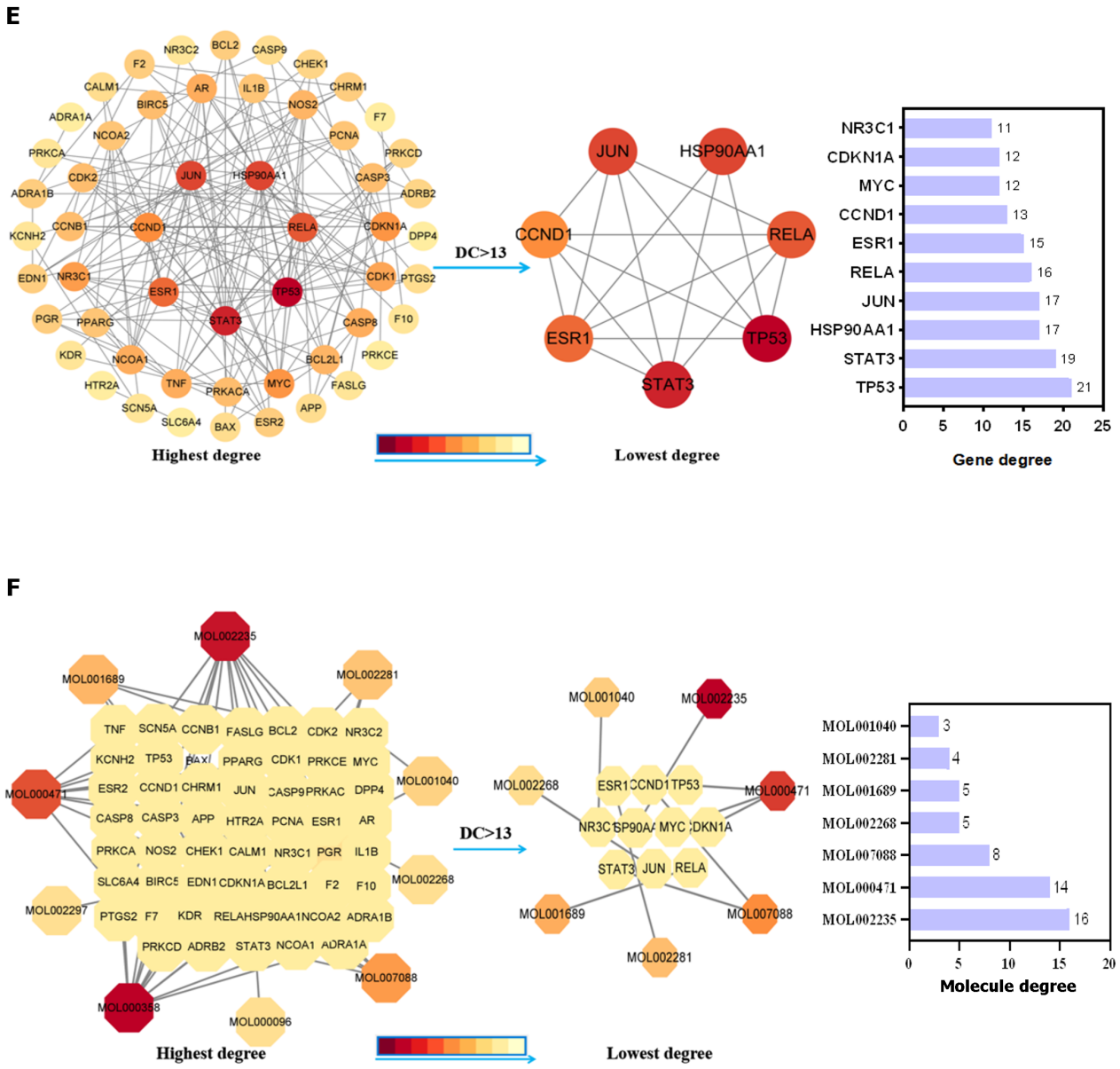


Figure 2 Identification of drug and disease targets and protein-protein interaction enrichment analysis. A: Drug and disease targets; B: Intersection of drug and disease targets; C: Drug-disease-target network diagram; D: Protein-protein interaction (PPI) network diagram of intersection genes of *Nardostachyos Radix et Rhizoma*-rhubarb drug pair (NRDP) and disease; E: PPI network containing 73 intersection targets and 10 core targets constructed using Cytoscape; F: Selected core targets and active components of NRDP were analyzed. Each node's color denotes the degree.

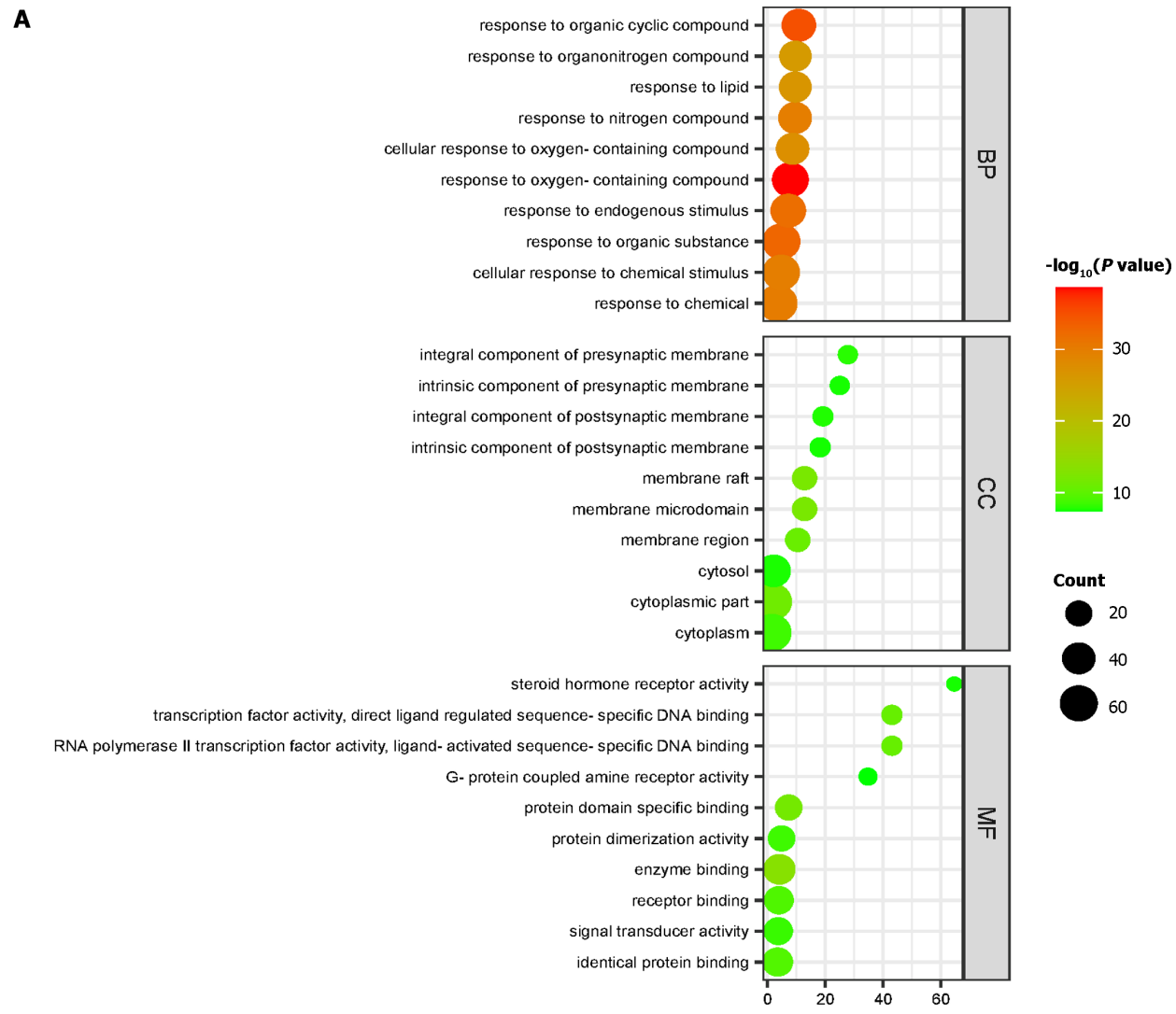
STRING11.5 database and Network Analyzer plug-in of Cytoscape software, as shown in Figure 3C. The signal pathway diagram was also generated (Figure 3D).

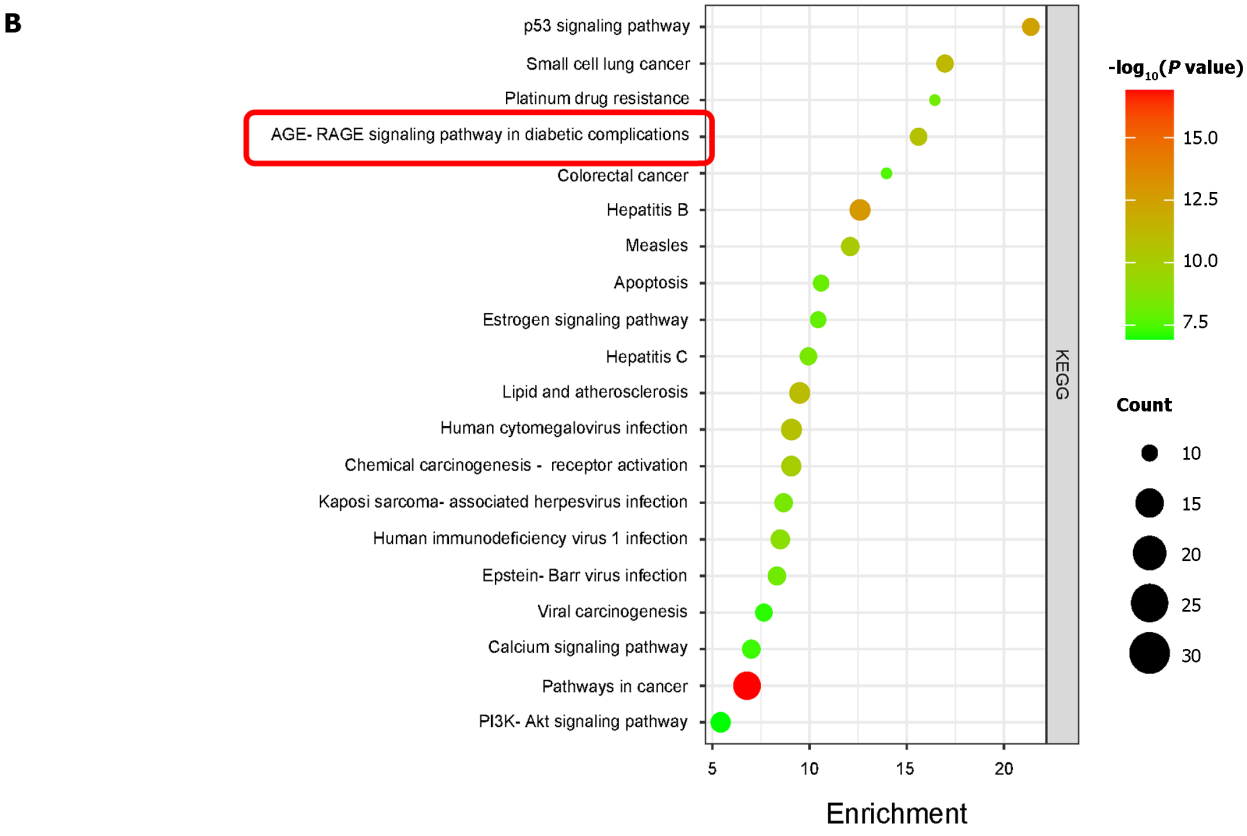
Molecular docking

To further analyze the feasibility of NRDP for the treatment of DKD, the core proteins TP53, STAT3, HSP90AA1, JUN, RELA, ESR1, and CCND1, which have top seven degree values, were molecularly docked with the active components of NRDP. The PDB ID of ESR1, RELA, TP53, HSP90AA1, JUN, CCND1, and STAT3 is 6CHW, 3CBQ, 3DCY, 1BYQ, 2P33, 2VTH, and 6NJS, respectively. Pymol software was used to visualize the docking results (Figure 4A). Binding activity was evaluated according to docking scores: Scores < -4.25 kcal/mol indicated low binding activity, scores < -5.0 kcal/mol indicated good binding activity, and scores < -7.0 kcal/mol indicated strong binding activity. ChiPlot (https://www.chiplot.online/#Heatmap) online tools were used for the visualization output (Figure 4B). The binding energy of the ten active ingredients with the seven core target proteins was all less than -5.0 kcal/mol, among which the binding energy of STAT3 and (-) -Catechin was the smallest at -9.3 kcal/mol.

Median inhibitory concentration of NRDP

To determine the inhibition rate of NRDP in each group, the OD values from the 24-h experiment were used for inhibition rate calculation. With GraphPad Prism 8.0.2, half-inhibitory doses for TCMK-1 cells were fitted (Figure 5A). According to


A
response to organic cyclic compound
response to organonitrogen compound
response to lipid
response to nitrogen compound
cellular response to oxygen- containing compound
response to oxygen- containing compound
response to endogenous stimulus
response to organic substance
cellular response to chemical stimulus
response to chemical
BP
integral component of presynaptic membrane
intrinsic component of presynaptic membrane
integral component of postsynaptic membrane
intrinsic component of postsynaptic membrane
membrane raft
membrane microdomain
membrane region
cytosol
cytoplasmic part
cytoplasm
CC
steroid hormone receptor activity
transcription factor activity, direct ligand regulated sequence- specific DNA binding
RNA polymerase II transcription factor activity, ligand- activated sequence- specific DNA binding
G- protein coupled amine receptor activity
protein domain specific binding
protein dimerization activity
enzyme binding
receptor binding
signal transducer activity
identical protein binding
MF
0
20
40
60
-log10(P value)
30
20
10
Count
20
40
60


B
p53 signaling pathway
Small cell lung cancer
Platinum drug resistance
AGE- RAGE signaling pathway in diabetic complications
Colorectal cancer
Hepatitis B
Measles
Apoptosis
Estrogen signaling pathway
Hepatitis C
Lipid and atherosclerosis
Human cytomegalovirus infection
Chemical carcinogenesis - receptor activation
Kaposi sarcoma- associated herpesvirus infection
Human immunodeficiency virus 1 infection
Epstein- Barr virus infection
Viral carcinogenesis
Calcium signaling pathway
Pathways in cancer
PI3K- Akt signaling pathway
KEGG
5
10
15
20
Enrichment
-log10(P value)
15.0
12.5
10.0
7.5
Count
10
15
20
25
30

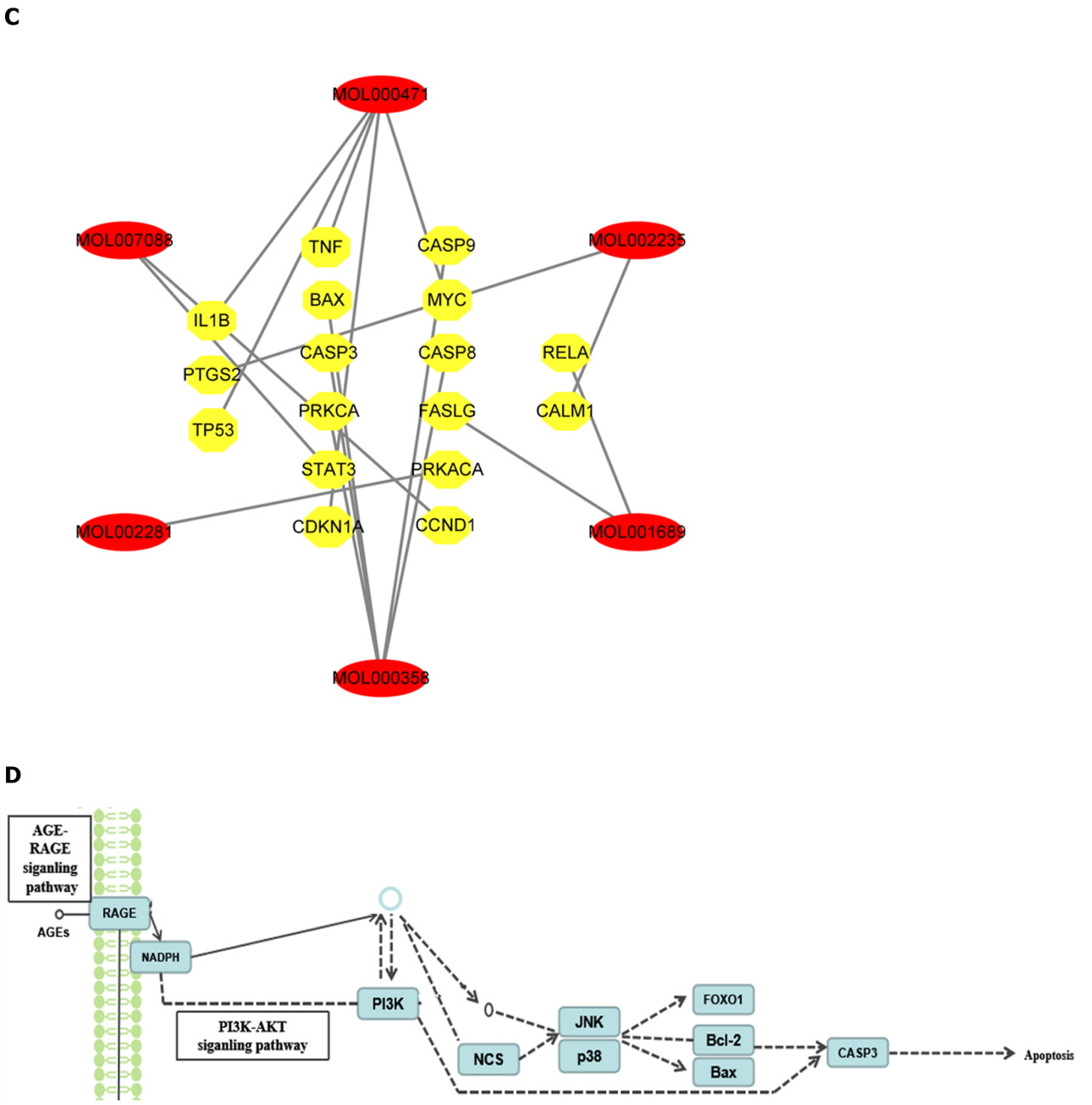


Figure 3 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis. A: Gene Ontology enrichment analysis; B: Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis; C: Enriched core genes in the AGE-RAGE signaling pathway; D: Correlation between AGE-RAGE signaling pathway and apoptosis.

the experimental results, the IC_{50} (half maximal inhibitory concentration) value of NRDP was 7.84 mg/mL. We determined that the half inhibitory concentration was 7 mg/mL, and the optimal low-, medium-, and high-does administration concentration was 4 mg/m, 7 mg/mL, and 10 mg/mL, respectively. Cell Counting Kit-8 assay showed that compared with that of the control group, the cell viability of the model group was significantly increased ($P < 0.01$). Compared with the model group, TCMK-1 cell viability decreased after NRDP intervention ($P < 0.01$) in a dose-dependent manner. The higher the NRDP dose, the more obvious the decline in TCMK-1 cell viability (Figure 5B).

Flow cytometry determination of effect of NRDP on the cell cycle of TCMK-1 cells induced by high glucose

After 24 h of NRDP intervention, flow cytometry was used to determine the cell cycle of TCMK-1 cells. Compared to cells in the control group (60.1 ± 0.70), cells of the model group showed an increase in the percentage of cells in the G0/G1 phase (40.23 ± 1.07; $P < 0.01$). Compared with the model group, the percentage of cells in the G0/G1 phase increased and that of the cells in the S phase decreased after NRDP intervention (NRDP-L: 45.55 ± 1.23, NRDP-M: 48.93 ± 0.75, NRDP-H: 61.04 ± 1.66; $P < 0.01$). Thus, NRDP could block TCMK-1 cells in the G0/G1 phase (Figure 5C).

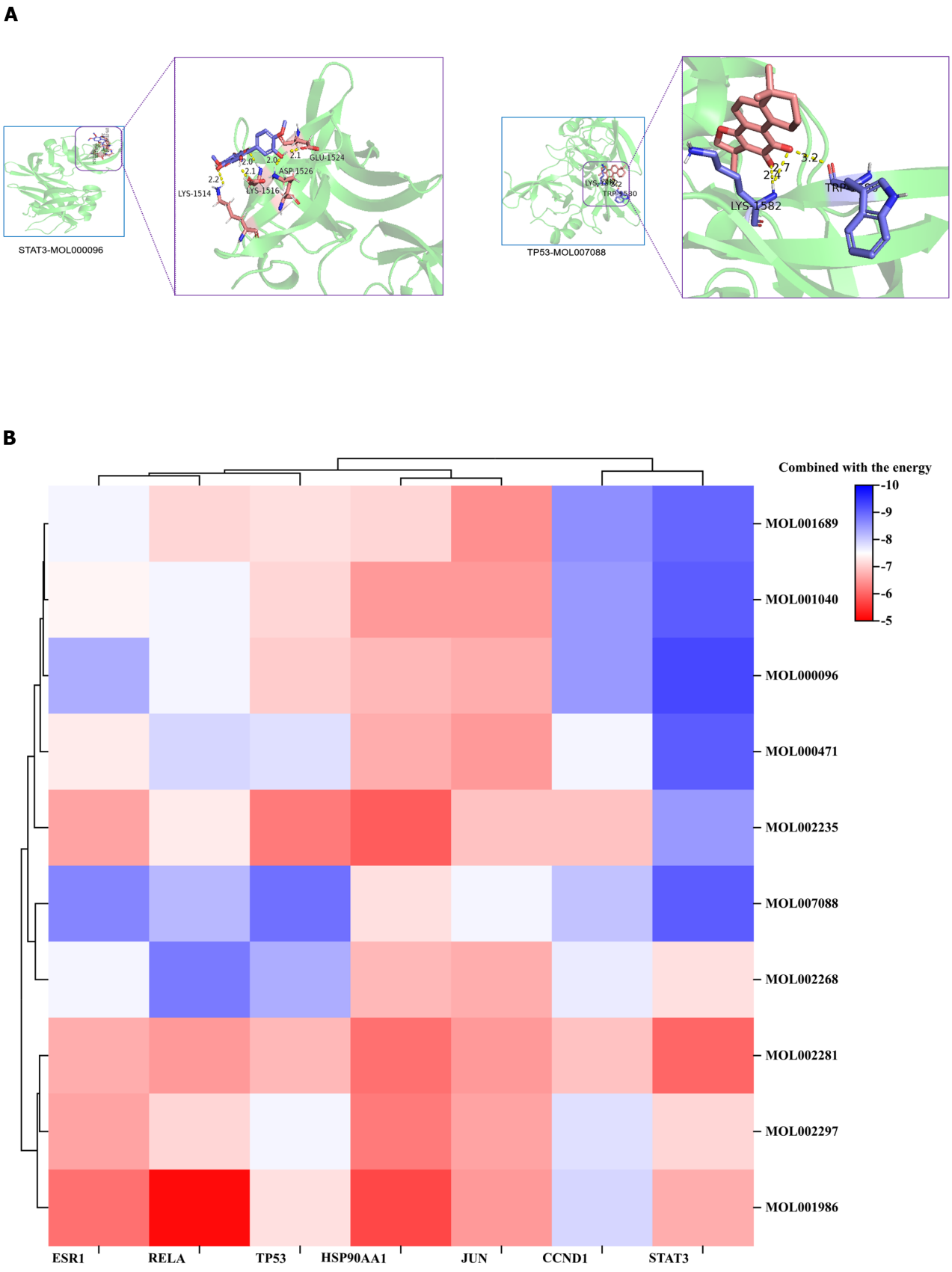
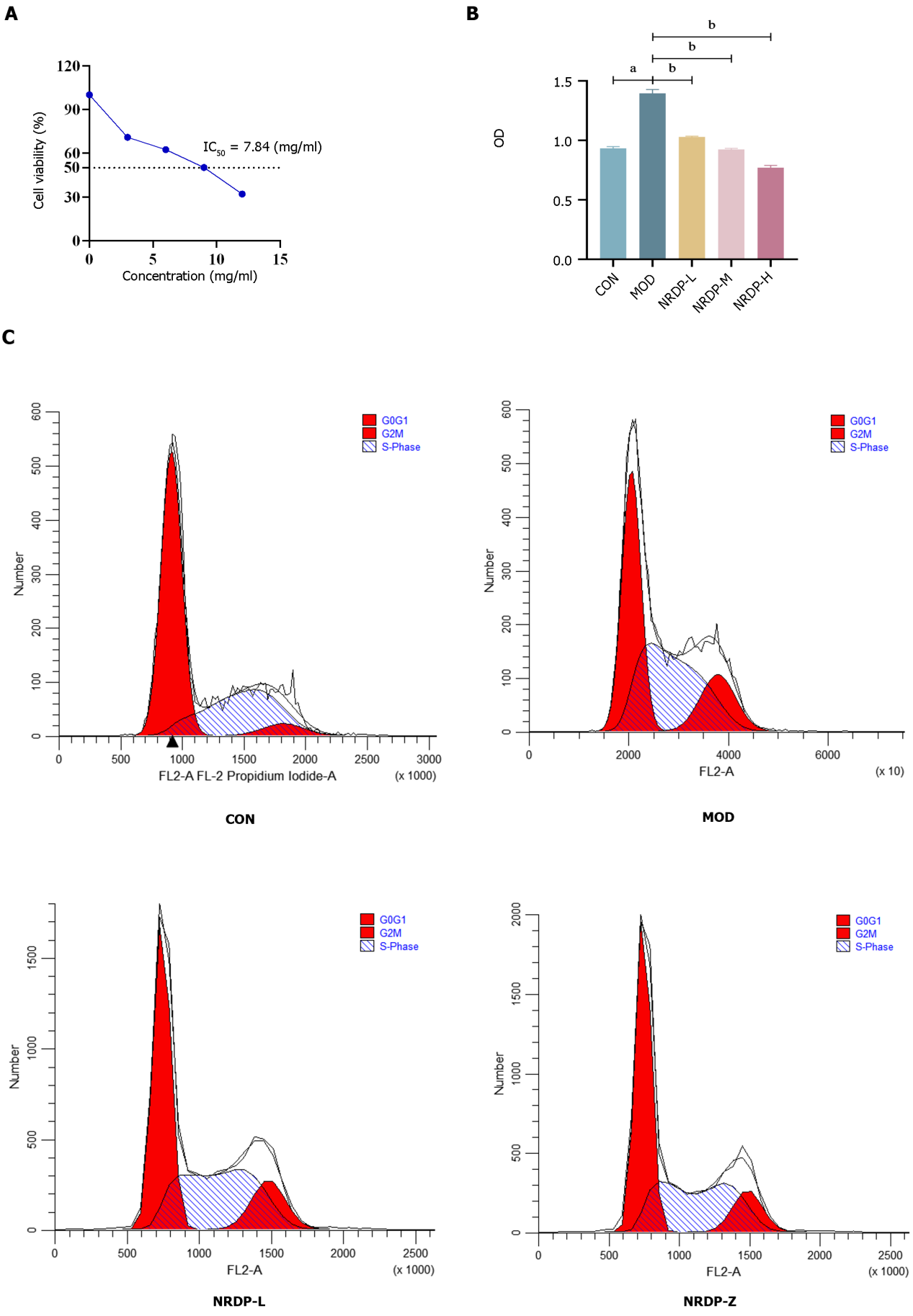
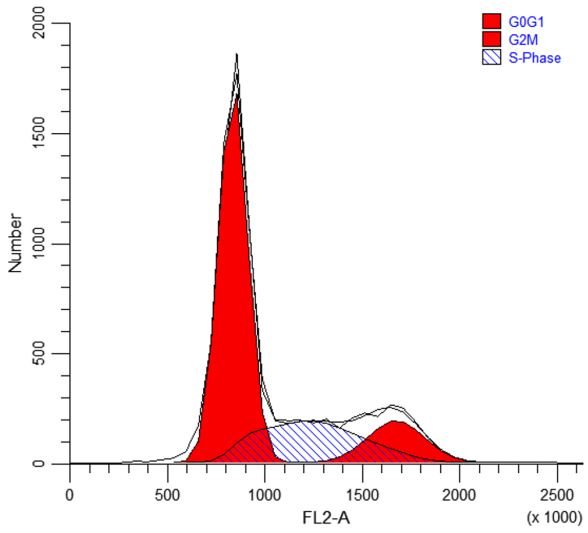
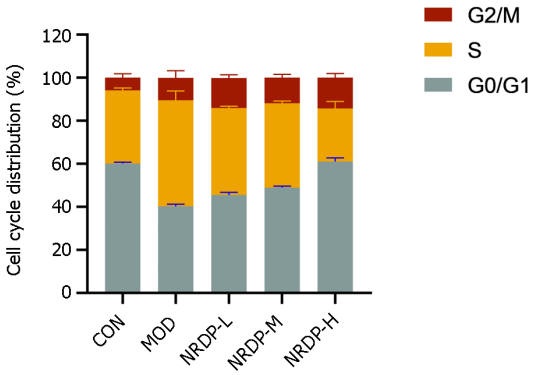


Figure 4 Molecules docking. A: Docking results between *Nardostachyos Radix et Rhizoma*-rhubarb drug pair components and core target molecules; B: Heat map of molecular docking showing the binding energy of core target molecules.

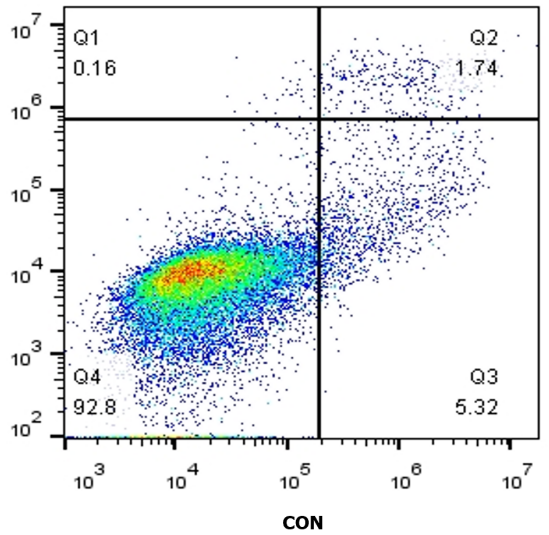

A
Cell viability (%)
120
90
60
50
30
0
IC50 = 7.84 (mg/ml)
0
5
10
15
Concentration (mg/ml)
B
OD
1.5
1.0
0.5
0.0
a
b
b
b
CON
MOD
NRDP-L
NRDP-M
NRDP-H
C
G0G1
G2M
S-Phase
Number
FL2-A FL-2 Propidium Iodide-A
(x 1000)
CON
FL2-A
(x 10)
MOD
NRDP-L
NRDP-Z



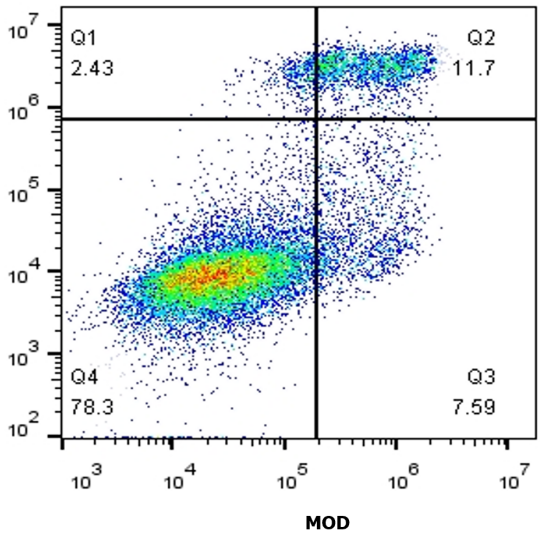
NRDP-H



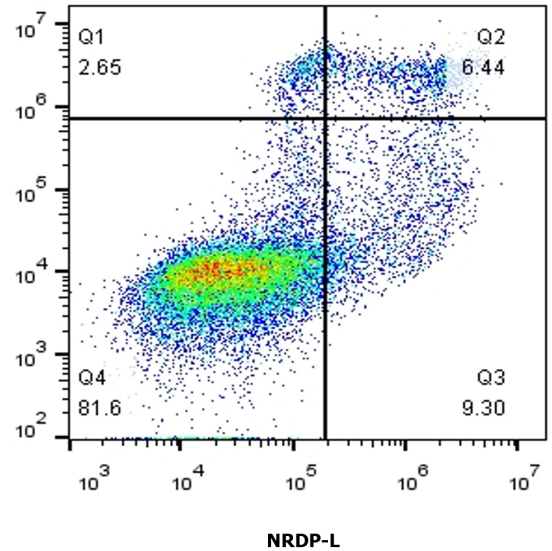
D



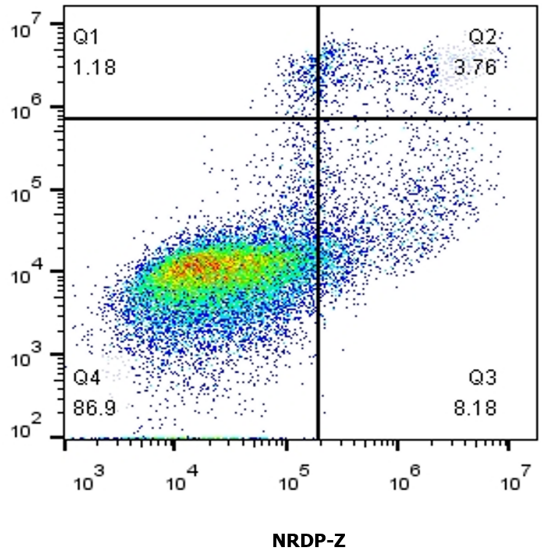
CON



MOD



NRDP-L



NRDP-Z

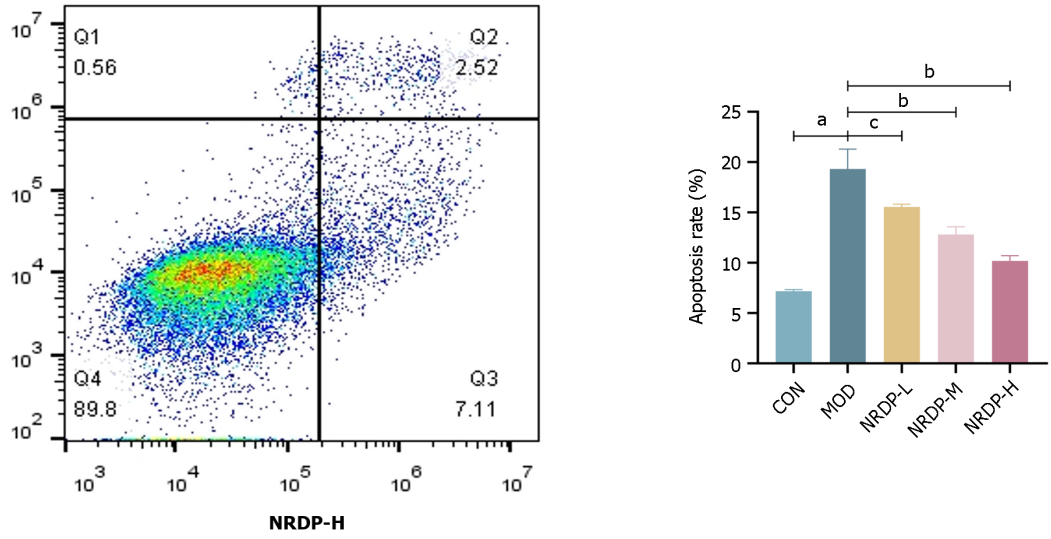


Figure 5 Effect of *Nardostachyos Radix et Rhizoma*-rhubarb drug pair on cellular phenotype of TCMK-1 cells. A and B: *Nardostachyos Radix et Rhizoma*-rhubarb drug pair (NRDP) reduces the activity of TCMK-1 cells; C: Effect of NRDP on the cell cycle of TCMK-1 cells; D: Effect of NRDP on apoptosis of TCMK-1 cells. CON: Control group; MOD: Model group; NRDP: *Nardostachyos Radix et Rhizoma*-rhubarb drug pair; IC_{50}: Half maximal inhibitory concentration. $^{a}P < 0.001$, $^{b}P < 0.001$, $^{c}P < 0.01$.

Flow cytometry detection of effect of NRDP on apoptosis of TCMK-1 cells induced by high glucose

Apoptosis of TCMK-1 cells was detected by AV-PI double staining and flow cytomety after 24 h of NRDP intervention. Compared with that of the control group (7.17 ± 0.168), the apoptosis rate of TCMK-1 cells in the model group was significantly increased (19.32 ± 1.975; $P < 0.01$). Compared with that of the model group, the apoptosis rate in the NRDP groups was decreased ($P < 0.01$), and with the increase of NRDP concentration, the apoptosis rate of TCMK-1 cells decreased more significantly (NRDP-L: 15.55 ± 0.257, NRDP-M: 12.80 ± 0.773, NRDP-H: 10.18 ± 0.523; Figure 5D).

Western blot analysis of expression of core proteins

Western blot was used to detect the expression of core proteins in TCMK-1 cells after NRDP intervention. The expression of p-STAT3, BAX, Caspase3, and Caspase9, as well as BAX/BCL-2 and p-STAT3/STAT3 ratios, was increased in the model group compared with the control group ($P < 0.01$), while the expression of BCL-2 and STAT3 proteins was decreased ($P < 0.01$). After intervention with NRDP, the expression of p-STAT3, BAX, Caspase3, and Caspase9, as well as BAX/BCL-2 and p-STAT3/STAT3 ratios, was decreased ($P < 0.01$ or $P < 0.05$), and the expression of BCL-2 and STAT3 proteins was increased ($P < 0.01$) (Figure 6).

DISCUSSION

This study investigated the mechanism of action of NRDP on DKD using network pharmacology. The results showed that NRDP had a therapeutic effect on DKD, with 10 active ingredients involving 85 targets. The GO, KEGG, and network interaction analyses revealed that NRDP may act on DKD through the AGE-RAGE signaling pathway (Figure 7). Our *in vitro* cell experiments confirmed that NRDP significantly inhibited TCMK-1 proliferation, promoted cell cycle arrest at the G0/G1 phase, and reduced the apoptosis of TCMK-1 cells in a dose-dependent manner. The results of the Western blot analysis indicated that NRDP intervention led to up-regulation of BCL-2 and STAT3 protein expression, and down-regulation of p-STAT3, BAX, Caspase3, and Caspase9 protein expression. Additionally, the BAX/BCL-2 and p-STAT3/STAT3 ratios were reduced. These findings suggest that NRDP is an effective treatment for DKD. NRDP protects renal tubular epithelial cells from high glucose-induced damage by regulating the AGE-RAGE signaling pathway.

AGEs are a group of complex molecules that form through non-enzymatic reactions between proteins or lipids and glucose or other carbohydrate derivatives. Their receptor RAGE is a multi-ligand receptor belonging to the immunoglobulin superfamily and is expressed in a wide range of tissues, including the vascular system, lung, heart, endothelial, and nervous tissues[23]. Their binding forms a key pathophysiological process associated with the occurrence and progression of many diseases, especially diabetes complications. AGEs from hyperglycemia interact with RAGE to activate many downstream effectors, including the JAK/STAT pathway, which in turn activates transcription factors like STAT3 over time[24]. This increases the inflammatory response and further exacerbates DKD[25]. Tang *et al*[26] showed through network pharmacology that the AGE-RAGE pathway is the most important pathway for *Coptis Jiedu* decoction to treat DKD, and *in vivo* experiments verified that *Coptis Jiedu* decoction can improve glucose and lipid metabolism disorder and kidney injury by regulating the AGEs-RAGE-AKT-Nrf2 pathway in db/db mice, thus playing a protective role in DKD. Hou *et al*[27] showed that salvianolic acid A inhibited AGEs-induced actin cytoskeletal rearrangement

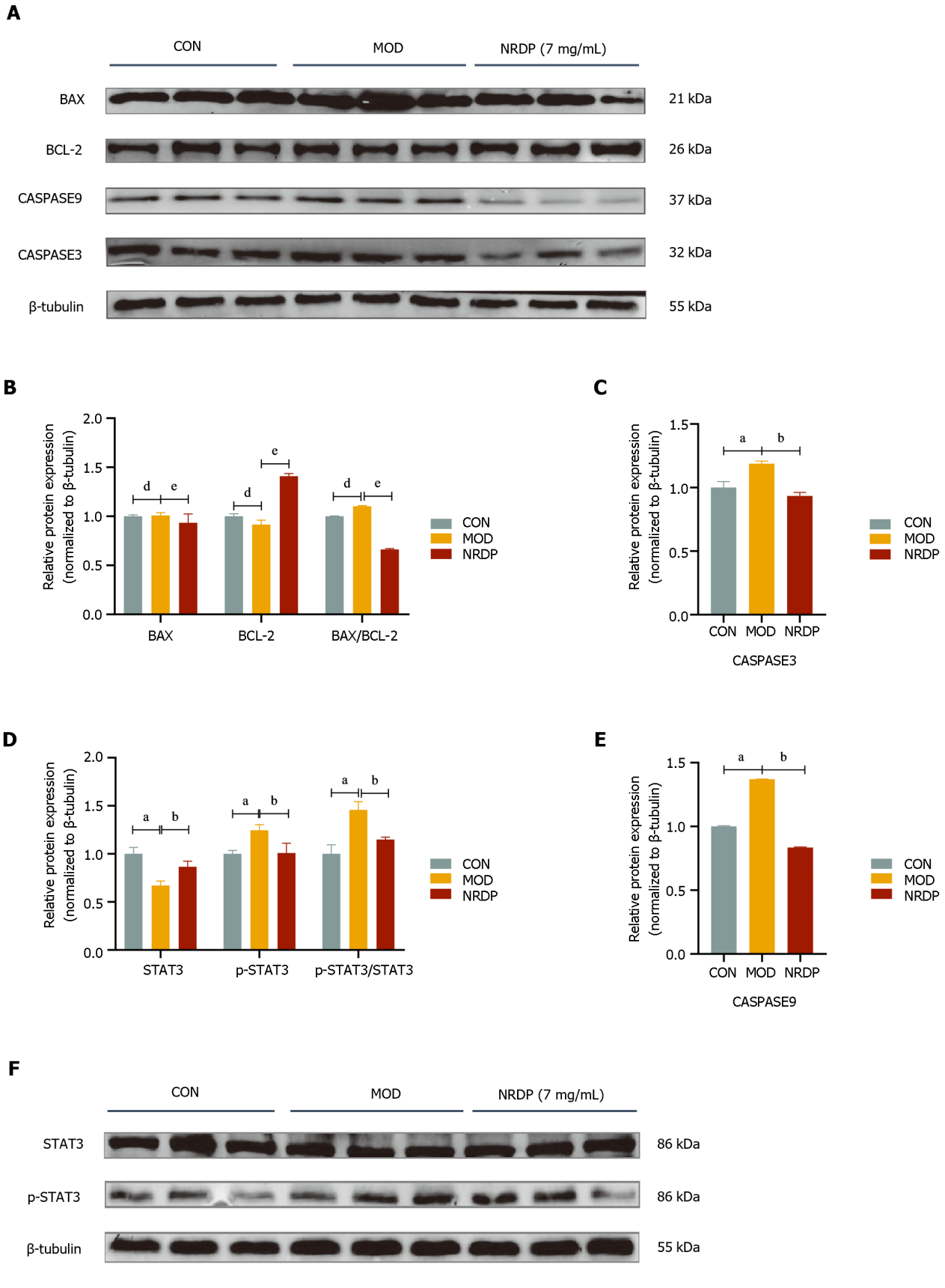


Figure 6 Effect of *Nardostachyos Radix et Rhizoma*-rhubarb drug pair on AGE-RAGE signaling pathway in TCMK-1 cells. A, B, C, and E: Protein expression of BAX, BCL-2, Caspase9, and Caspase3; D and F: Expression of STAT3 and p-STAT3 protein. CON: Control group; MOD: Model group; NRDP: *Nardostachyos Radix et Rhizoma*-rhubarb drug pair. [a]*P* <0.001, [b]*P* < 0.001, [c]*P* <0.01, [d]*P* < 0.05, [e]*P* <0.05.

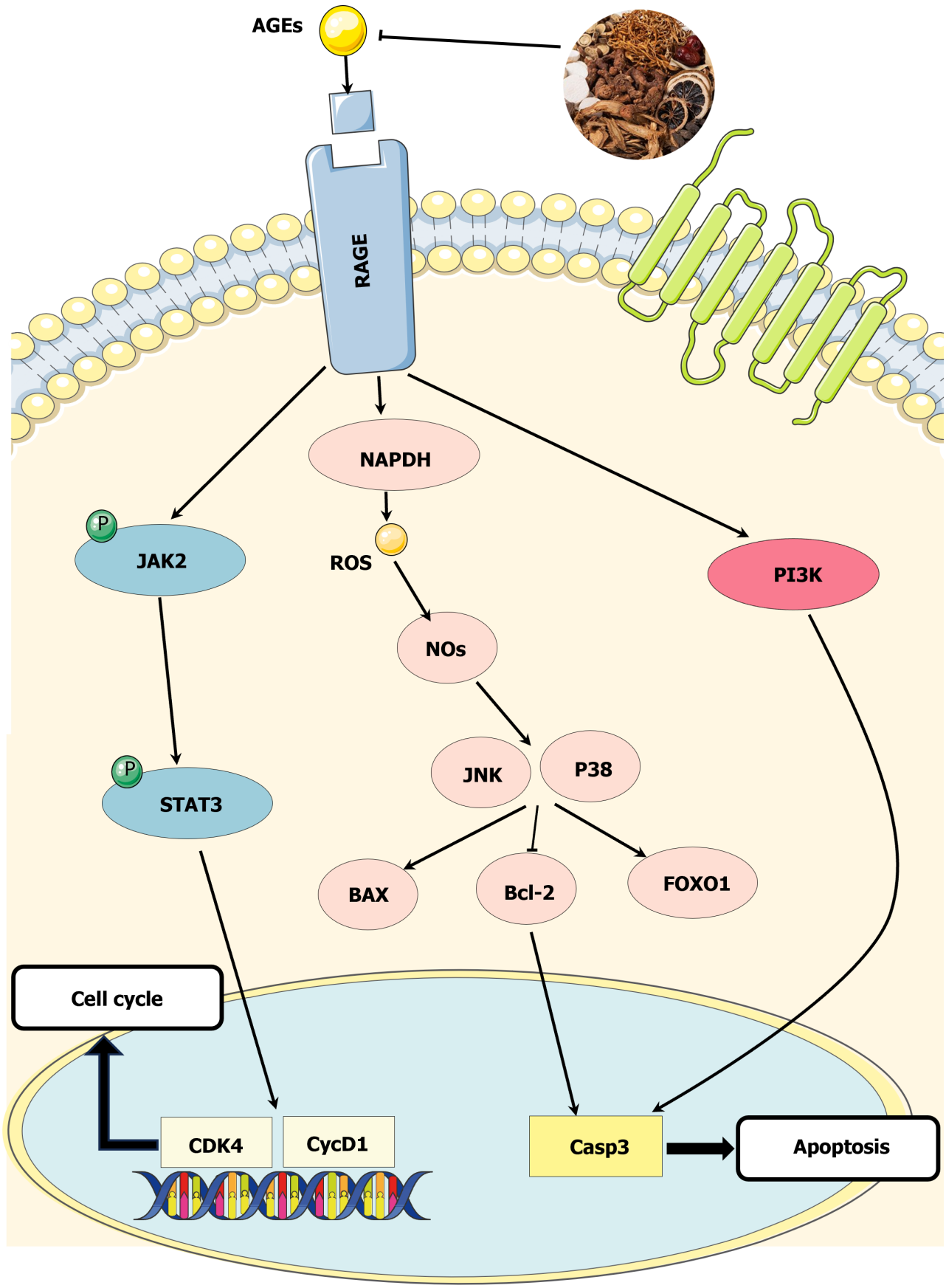


Figure 7 Mechanism of *Nardostachyos Radix et Rhizoma*-rhubarb drug pair in treatment of diabetic kidney disease. NAGE: Receptor for advanced glycation end products; ROS: Reactive oxygen species.

through the AGEs-RAGE-Rhoa-Rock pathway, restored glomerular endothelial permeability, weakened AGEs-induced oxidative stress, restored glomerular endothelial function, alleviated renal structural deterioration, and effectively improved early DKD. The changes in the expression of relevant proteins after NRDP intervention in this study showed that the drug alleviated DKD symptoms to some extent.

Numerous studies have demonstrated that the production of AGEs linked to hyperglycemia is a key factor in the pathophysiology of DKD. The RAGE binds to its ligands, inducing oxidative stress and chronic inflammation in renal tissue, ultimately resulting in renal dysfunction. AGEs can alter the extracellular matrix by involving cell surface receptors and producing proinflammatory cytokines. RAGE and its ligands promote angiogenesis, cell migration, proliferation, invasion, and metastasis by limiting apoptotic cell death[28]. Studies have shown that AGEs and their receptor RAGE can induce apoptosis in different cell types. The propagation of apoptosis through the AGE-RAGE signaling pathway involves the cascade reaction of the pro-apoptotic factor, which prompts the apoptotic signal to activate the apoptotic factor Caspase3[29] and initiates the occurrence of apoptosis. Under the influence of certain receptors and factors, the endogenous apoptotic pathway is activated and regulated by the BCL-2 protein, which directly activates Caspase9. The Caspase cascade can activate Caspase3 during apoptosis induced by death receptors and DNA damage, producing intracellular signals that act on cellular targets, ultimately leading to programmed cell death[30]. Previous studies have demonstrated that RAGE expression regulates apoptotic death receptors and mitochondrial pathways by controlling the expression of pro-apoptotic Caspase3, Caspase9, and anti-apoptotic BCL-2. BAX, a proapoptotic protein, and BCL-2, a regulatory protein of apoptosis, can form Bax-Bcl-2 heterodimers when BCL-2 binds to active BAX protein in the cytoplasm, thus playing a role in reducing apoptosis. Reducing the activity of the BAX protein can also negatively regulate apoptosis. The amount of apoptosis can be determined by the degree of binding between BAX and BCL-2. Reducing the activity of BAX and promoting the binding of BCL-2 to BAX protein can reduce apoptosis[31]. Our study found that the expression of apoptosis-related proteins in TCMK-1 cells was detected after the intervention of NRDP. The expression of BAX, Caspase3, and Caspase9 proteins was downregulated, while the expression of BCL-2 protein was upregulated. This may be due to the induction of BCL-2 expression by NRDP. The inhibition of BAX protein activity resulted in a weakened Caspase family cascade and reduced apoptosis of renal tubular epithelial cells. This illustrates the pharmacological effect of NRDP in treating DKD.

In summary, NRDP may prevent TCMK-1 cells from proliferating and reduce cell death by controlling the relevant proteins of the AGE-RAGE signaling pathway, thereby protecting the function of intrinsic kidney cells during high glucose levels. Currently, there are numerous studies on the pathogenesis of DKD, which can be summarized as the result of a combination of metabolic, inflammatory, hemodynamic, and fibrotic factors. Many scholars have explored the treatment of DKD. Some treatments targeting specific pathogenic mechanisms are often used in clinical and experimental studies. Combination therapies involving two or more drugs have been found to have the potential to treat DKD. For instance, combining ERA with SGLT2 inhibitors has shown promise[32]. The present study also validated the efficacy of a herbal combination for treating DKD, providing a preliminary possibility for future exploration of new combinations of traditional Chinese medicine combined with other inhibitors and drugs for treating DKD. However, this study was only limited to *in vitro* cellular experiments due to funding constraints. Our group's research on treating DKD with traditional Chinese medicine is ongoing, and we plan to incorporate high-throughput histological methods for further validation in the future. We will use high-throughput genomics methods for the validation and identification of a safe and effective clinical treatment for DKD, which will improve the prognosis and quality of life of such patients.

CONCLUSION

In this study, TCMK-1 cells were treated with varying concentrations of NRDP in a hyperglycemic environment. The results indicated that NRDP can regulate the cell cycle of TCMK-1 cells by blocking them in the G0/G1 phase, affecting the process from the late stage of DNA synthesis to the completion of mitosis and reducing apoptosis in a dose-dependent manner. Additionally, NRDP may upregulate the expression of BCL-2 and STAT3. The expression of p-STAT3, BAX, Caspase3, and Caspase9 proteins was downregulated, as well as the BAX/BCL-2 and p-STAT3/STAT3 ratios. Consequently, the impaired AGE-RAGE signal axis has a greater impact on the body during high glucose conditions, and the high glucose environment has a protective effect on renal tubular epithelial cells. This lays the foundation for the search for safe and effective drugs to treat DKD.

ARTICLE HIGHLIGHTS

Research background

Diabetic kidney disease (DKD) is one of the serious complications of diabetes mellitus. It has a poor prognosis and is one of the causes of end-stage renal disease. Existing treatments can improve the symptoms of DKD to some extent. However, they have the disadvantages of side effects and high price.

Research motivation

We performed *in vitro* cellular experiments to validate the effectiveness of the *Nardostachyos Radix et Rhizoma*-rhubarb drug pair (NRDP) and to provide new ideas for clinical treatment of DKD.

Research objectives

In this study, we used network pharmacology and molecular docking to predict the targets of NRDP for the treatment of DKD and validated the prediction findings using cellular experiments.

Research methods

Targets for NRDP and DKD were obtained using databases such as TCMSP, Genecards, OMIM, and TTD. Drug-disease intersection targets were obtained based on the VENNY 2.1 database and "drug-component-target-disease" network was constructed. Afterward, Kyoto Encyclopedia of Genes and Genomes pathway and Gene Ontology enrichment analyses were performed to further observe the relationship between targets and pathways. Finally, molecular docking was performed on the active ingredients of NRDP. Experiments such as the CCK-8 method, flow cytometry, and Western Blot were used to verify the molecular mechanism of NRDP for DKD.

Research results

NRDP may inhibit the viability of high glucose-induced TCMK-1 cells by modulating the advanced glycation end products (AGEs)-receptor for AGEs (RAGE) signaling pathway, thereby blocking cell cycle progression in the G0/G1 phase and reducing apoptosis. It also downregulated the protein expression of p-STAT3, BAX, Caspase3, and Caspase9, and up-regulated the protein levels of BCL-2 and STAT3. These findings verified that NRDP could reduce high glucose-induced TCMK-1 cell injury, thereby restoring their function.

Research conclusions

NRDP may achieve its therapeutic effect on DKD by modulating the AGE-RAGE signaling pathway. NRDP arrests the cell cycle progression at the G0/G1 phase by inhibiting the proliferation of high glucose-induced TCMK-1 cells and reducing their apoptosis. NRDP inhibits the expression of proteins related to the AGE-RAGE signaling pathway in high glucose environment, which delays the progression of DKD.

Research perspectives

We next plan to conduct *in vivo* animal and omics experiments. To determine the specific components of NRDP in the blood for the treatment of DKD, gene detection will be performed by high-throughput validation methods such as transcriptomics, in order to provide a safe and effective method for clinical treatment of DKD.

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FOOTNOTES

Author contributions: Che MY conducted most of the experiments, analyzed the data, completed the figure production, and wrote the manuscript; Min J carried out a portion of the experiments and participated in the production of the figures and the composition of the manuscript; Xu DJ and Liu WJ carried out part of the experiments and participated in the statistical analysis of the data; Lu DD and Wang KL performed the network pharmacology prediction; Yuan L and Wang YY designed the study; Nan Y revised and improved the manuscript; all authors approved the final version of the article.

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Data sharing statement: No additional data are available.

Country/Territory of origin: China

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SYSTEMATIC REVIEWS

KCNQ1 rs2237895 gene polymorphism increases susceptibility to type 2 diabetes mellitus in Asian populations

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Abstract

BACKGROUND

The association of single nucleotide polymorphism of *KCNQ1* gene rs2237895 with type 2 diabetes mellitus (T2DM) is currently controversial. It is unknown whether this association can be gene realized across different populations.

AIM

To determine the association of *KCNQ1* rs2237895 with T2DM and provide reliable evidence for genetic susceptibility to T2DM.

METHODS

We searched PubMed, Embase, Web of Science, Cochrane Library, Medline, Baidu Academic, China National Knowledge Infrastructure, China Biomedical Literature Database, and Wanfang to investigate the association between *KCNQ1* gene rs2237895 and the risk of T2DM up to January 12, 2022. Review Manager 5.4 was used to analyze the association of the *KCNQ1* gene rs2237895 polymorphism with T2DM and to evaluate the publication bias of the selected literature.

RESULTS

Twelve case–control studies (including 11273 cases and 11654 controls) met our inclusion criteria. In the full population, allelic model [odds ratio (OR): 1.19; 95% confidence interval (95%CI): 1.09–1.29; $P < 0.0001$], recessive model (OR: 1.20; 95%CI: 1.11–1.29; $P < 0.0001$), dominant model (OR: 1.27. 95%CI: 1.14–1.42; $P <$

0.0001), and codominant model (OR: 1.36; 95%CI: 1.15–1.60; *P* = 0.0003) (OR: 1.22; 95%CI: 1.10–1.36; *P* = 0.0002) indicated that the *KCNQ1* gene rs2237895 polymorphism was significantly correlated with susceptibility to T2DM. In stratified analysis, this association was confirmed in Asian populations: allelic model (OR: 1.25; 95%CI: 1.13–1.37; *P* < 0.0001), recessive model (OR: 1.29; 95%CI: 1.11–1.49; *P* = 0.0007), dominant model (OR: 1.35; 95%CI: 1.20–1.52; *P* < 0.0001), codominant model (OR: 1.49; 95%CI: 1.22–1.81; *P* < 0.0001) (OR: 1.26; 95%CI: 1.16–1.36; *P* < 0.0001). In non-Asian populations, this association was not significant: Allelic model (OR: 1.06, 95%CI: 0.98–1.14; *P* = 0.12), recessive model (OR: 1.04; 95%CI: 0.75–1.42; *P* = 0.83), dominant model (OR: 1.06; 95%CI: 0.98–1.15; *P* = 0.15), codominant model (OR: 1.08; 95%CI: 0.82–1.42; *P* = 0.60. OR: 1.15; 95%CI: 0.95–1.39; *P* = 0.14).

CONCLUSION

KCNQ1 gene rs2237895 was significantly associated with susceptibility to T2DM in an Asian population. Carriers of the C allele had a higher risk of T2DM. This association was not significant in non-Asian populations.

Key Words: Type 2 diabetes mellitus; KCNQ1; rs2237895; Single nucleotide polymorphism; Asian populations

Core Tip: In Asian populations, the rs2237895 polymorphism in the *KCNQ1* gene was significantly associated with susceptibility to type 2 diabetes mellitus (T2DM), and C allele carriers had an increased risk of developing T2DM. The CC and AC genotypes of *KCNQ1* rs2237895 significantly increased the susceptibility to T2DM. In non-Asian populations, this association was not significant.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a common multifactorial, metabolic disease whose pathogenesis is influenced by a combination of genetic and environmental factors. The rise and large-scale application of genome-wide association studies have contributed to the understanding of genetic factors related to T2DM. T2DM remains a health problem that plagues the world to this day. As of January 4, 2021, the number of people with diabetes worldwide had reached 537 million. Even more alarmingly, this number is expected to increase to 643 million by 2030. The various expenditures due to diabetes have exceeded $966 billion, and this figure has grown at an annual rate of 63% since 2006[1]. The etiology of T2DM is complex and has not yet been fully elucidated. T2DM is characterized by defective insulin secretion and reduced sensitivity, leading to chronic hyperglycemia and severe metabolic dysfunction in patients[2,3]. Hyperglycemia affects the physiological function of several tissues and organs in the body, among which the most common are neuropathy and vascular complications[1].

Studies have not provided an accurate description of the etiology of T2DM, and a genome-wide scan of Japanese by Nawata *et al*[4] showed that *KCNQ1* is a susceptibility gene for T2DM in Japan. In addition, genes such as *ADRA2A, KCNJ11 and CDKAL1* may be associated with the development of T2DM[4,5]. *KCNQ1* is a potassium channel subunit that is mainly found in adipose and pancreatic tissues. It was found that *KCNQ1* affects the process of islet β-cell depolarization by regulating potassium channel currents, thereby limiting insulin secretion from pancreatic β-cells and leading to the development of T2DM[6].

Previous studies have found that C allele carriers of the *KCNQ1* gene rs2237895 may have an increased risk of developing T2DM[7]. rs2237895 is present in three genotypes in the population, AA, AC and CC. The A gene is wild type and the C gene is mutant, and their gene frequencies in the population are approximately 66% and 34%[8]. A study by Cui *et al*[7] in Kazakhs living in China showed that rs2237895 single nucleotide polymorphism (SNP) of *KCNQ1* gene was not significantly associated with T2DM. A study by Afshardoost *et al*[9] on Iranians also showed no significant association between rs2237895 and T2DM; while in a study by Khan *et al*[10] on Indians, they confirmed a significant association between the SNP of rs2237895 and T2DM. Previously, a similar study has been conducted by Sun *et al*[11], but we consider that their inclusion criteria were more lenient and the strength of the proof may be weakened. Meanwhile, their work was > 10 years old and many new studies have been published during this period and that meta-analysis is in urgent need of updating. To address the above issues, we performed the present meta-analysis.

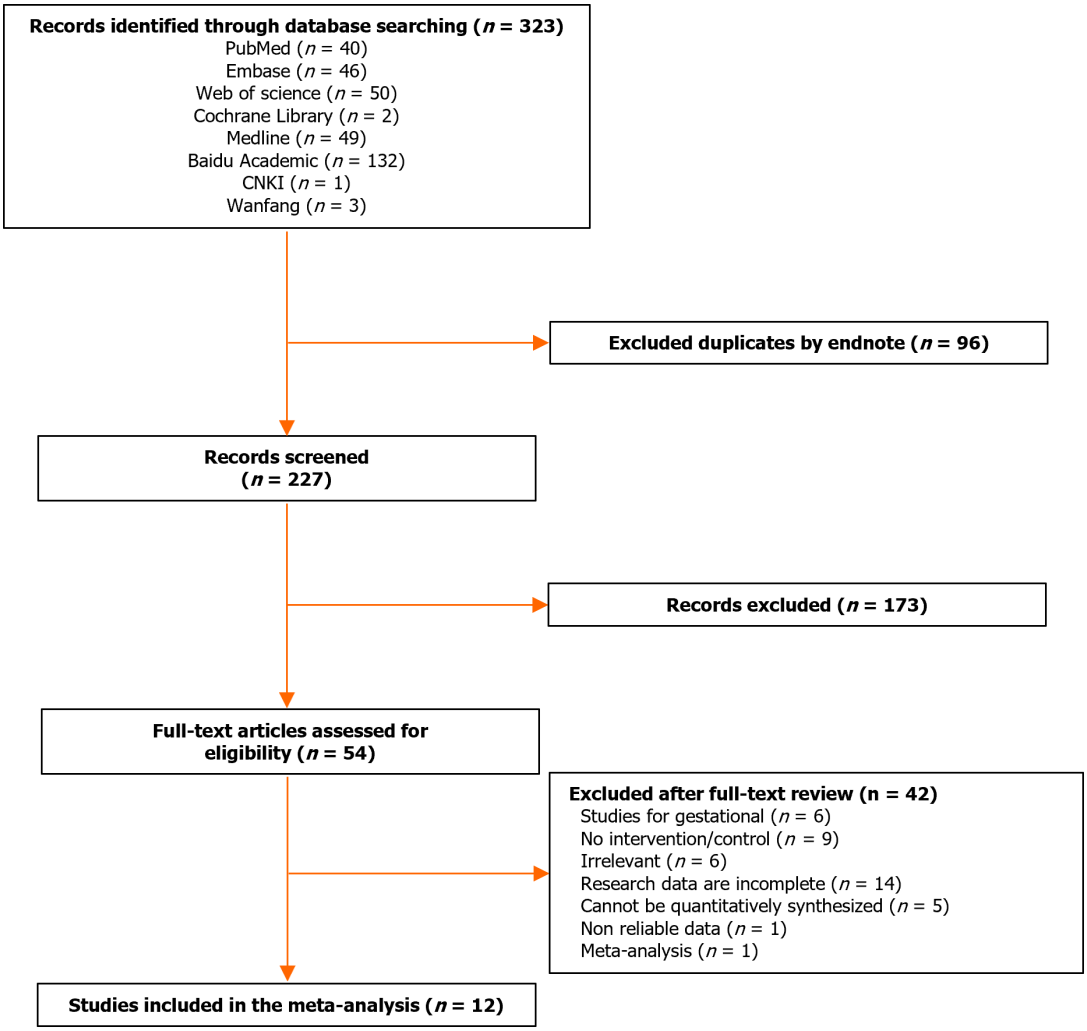


Figure 1 Literature screening process.

MATERIALS AND METHODS

Literature search

The following nine electronic databases were searched: PubMed, Embase, Web of Science, Cochrane Library, Medline, Baidu Academic, China National Knowledge Infrastructure (CNKI), China Biomedical Literature Database (CBM), and Wanfang Database, with the following search formulas: Subject (T2DM) and keywords (KCNQ1) and keywords (rs2237895). The last search date was January 12, 2022. Chinese and English literature on the association of the rs2237895 SNP in the *KCNQ1* gene with T2DM was collected. The inclusion criteria for the articles were: (1) T2DM patients in the case group met the diagnostic criteria for diabetes published by WHO in 1999 or American Diabetes Association in 2010; (2) the type of experiment was a case–control study or a cohort study; (3) there was sufficient information in the text to describe the genotype and allele frequencies of the case and control groups; (4) the patients in the control group all met the Hardy–Weinberg genetic equilibrium model; (5) patients were randomly selected with no special restrictions on age, sex, or family history; and (6) for duplicate or data-identical literature, the one with the most complete information. Exclusion criteria were: (1) Incomplete study data; (2) literature reviews; (3) studies with gestational diabetes as an endpoint; and (4) exclusion of studies with familial diabetes as a basis.

Data extraction

Two researchers independently performed literature screening and extraction of information based on the above criteria. A third researcher was required to discuss and agree on the results when difficult differences were encountered. For each article, we collected the basic information that needed to be used for Meta-analysis, and the literature screening process is shown in Figure 1.

Statistical analysis

The data were processed using Review Manager 5.4. The strength of association between SNPs in the *KCNQ1* gene rs2237895 and the risk of T2DM was assessed using the odds ratio (OR) and its corresponding 95% confidence interval (95%CI) as a criterion in the data statistics. The forest plots were used to show the OR and its 95%CI for each study. The

Table 1 Characteristics of the included literature

Ref.	Ethnicity	*N*		Age (yr)		Case			Control		
		Case	Control	Case	Control	AA	AC	CC	AA	AC	CC
Cui *et al*[7], 2016	Kazakh	100	100	51.21 ± 11.60	49.85 ± 12.41	40	49	11	32	51	17
Khan *et al*[10], 2020	Indian	300	100	40.33 ± 9.76	35.29 ± 7.96	90	153	57	50	36	14
Liu *et al*[12], 2009	Chinese	1885	1994	63.9 ± 9.50	58.10 ± 9.40	790	886	209	942	883	169
Zhang[13], 2010	Chinese	100	97	63.90 ± 9.50	58.1 ± 9.40	25	36	39	43	34	20
Dai *et al*[14], 2012	Chinese	367	214	49.13 ± 10.79	47.55 ± 10.93	134	168	65	99	87	28
Li *et al*[15], 2020	Chinese	1194	1292	52.49 ± 12.10	52.70 ± 10.52	509	568	117	621	552	119
Hu *et al*[16], 2021	Chinese	277	279	52.26 ± 9.49	52.26 ± 9.49	121	123	33	145	113	21
Saif-Ali *et al*[17], 2011	Malaysian	300	230	49.80 ± 7.42	52.90 ± 9.15	123	147	30	120	96	14
Almawi *et al*[18], 2013	Arabs	995	1076	58.6 ± 13.40	57.30 ± 10.40	324	497	174	413	511	152
Al-Shammari *et al*[19], 2017	Arabs	320	516	51.50 ± 8.75	48.75 ± 6.85	122	150	58	202	223	91
van Vliet-Ostaptchouk *et al*[20], 2012	Dutch	4549	5182	64.36 ± 10.6	51.16 ± 10.10	1522	2158	869	1803	2516	863
Turki *et al*[21], 2012	Arabs	886	574	61.20 ± 9.70	52.00 ± 11.9	350	429	107	233	261	80

pooled results were directly observed on the forest plots. The difference was considered significant when the 95%CI did not include 1. Allelic model (C *vs* A), recessive model (CC *vs* AA + AC), dominant model (CC + AC *vs* AA) and codominant model (CC *vs* AA and AC *vs* AA) were used to assess the genetic effects of the genes. The significance level was set at $P < 0.05$. The random-effect model was used to calculate the effect size when the heterogeneity was $I^2 > 50\%$, and the fixed-effect model was used when I^2 was < 50%. Publication bias was assessed by Egger's test and funnel plot. In the funnel plot, the dashed line perpendicular to the horizontal axis indicated the combined effect size. It suggested that the studies were without publication bias when the distribution of studies in the funnel plot was approximately symmetrical.

RESULTS

According to the research strategy, 323 relevant papers were retrieved from the databases. Some duplicates were found and we removed them by Endnote software. We also screened the citations of the paper to ensure the comprehensiveness of the search. After a stepwise screening process, 12 eligible papers were finally included for meta-analysis, which included 11273 patients with T2DM and 11654 controls. Five of the datasets were from China[12-16], five from the rest of Asia[7,10,17-19], one from Europe[20], and one from Africa[21]. The basic information of the studies is shown in Table 1.

The 12 datasets that met the inclusion criteria were pooled for meta-analysis, and allelic, recessive, dominant, and codominant models were used to investigate the association of rs2237895 with T2DM. Since the study population was predominantly Asian, we performed stratified analysis of Asian and non-Asian populations (Figure 2 and Figure 3).

In the full population, allelic model (OR: 1.19; 95%CI: 1.09–1.29; $P < 0.0001$), recessive model (OR: 1.20; 95%CI: 1.11–1.29; $P < 0.0001$), dominant model (OR: 1.27. 95%CI: 1.14–1.42; $P < 0.0001$), and codominant model (OR: 1.36; 95%CI: 1.15–1.60; $P = 0.0003$. OR: 1.22; 95%CI: 1.10–1.36; $P = 0.0002$) all showed significant association between rs2237895 and T2DM. In the subgroup of the Asian population, allelic model (OR: 1.25; 95%CI: 1.13–1.37; $P < 0.0001$), recessive model (OR: 1.29; 95%CI: 1.11–1.49; $P = 0.0007$), dominant model (OR: 1.35; 95%CI: 1.20–1.52; $P < 0.0001$), and codominant model (OR: 1.49; 95%CI: 1.22–1.81; $P < 0.0001$. OR: 1.26; 95% CI: 1.16–1.36; $P < 0.0001$) also showed a significant association between rs2237895 and T2DM, which was consistent with the whole population. C allele carriers had an increased risk of developing T2DM. The CC and AC genotypes significantly increased the risk of T2DM compared to the AA genotype. However, in the non-Asian population subgroup, allelic model (OR: 1.06; 95%CI: 0.98–1.14; $P = 0.12$), recessive model (OR: 1.04; 95%CI: 0.75–1.42; $P = 0.83$), dominant model (OR: 1.06; 95%CI: 0.98–1.15; $P = 0.15$), and codominant model (OR: 1.08; 95%CI: 0.82–1.42; $P = 0.60$) (OR: 1.15; 95%CI: 0.95–1.39; $P = 0.14$) all showed no significant association between rs2237895 and T2DM.

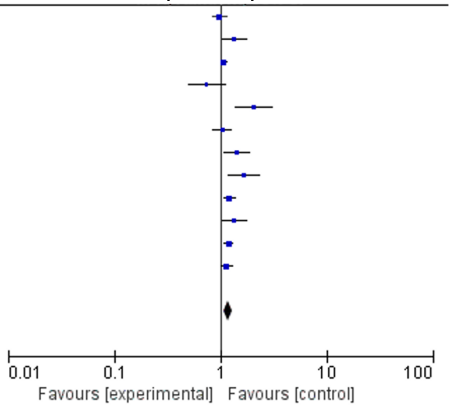
The funnel plots showed no significant publication bias was found in the meta-analysis (Figure 4 and Figure 5). Egger's test showed no significant publication bias for the allelic model ($t = 1.84$, $P = 0.095$), recessive model ($t = 0.48$, $P = 0.64$), dominant model ($t = 1.44$, $P = 0.18$), and codominant model ($t = 1.33$, $P = 0.21$; $t = 1.79$, $P = 0.10$).

We performed a sensitivity analysis. After sequentially excluding one study in the allelic model, recessive model, dominant model, and codominant model, we calculated the pooled effect sizes for the remaining studies. By calculation, no qualitative change occurred between the pooled results of the remaining studies and the original results. Sensitivity analysis proved that the results of the meta-analysis were reliable.

A

Study or subgroup	T2DM Events	T2DM Total	Control Events	Control Total	Weight	Odds ratio M-H, random, 95%CI
Amira Turki 2012	643	1772	421	1148	10.2%	0.98 [0.84, 1.15]
Fulan Hu 2021	189	554	155	558	6.4%	1.35 [1.04, 1.74]
Jana V. van Vliet-Ostaptchouk 2012	3896	9098	4242	10364	14.7%	1.08 [1.02, 1.14]
L.J. Cui 2016	71	200	85	200	3.4%	0.74 [0.50, 1.11]
Li Zhang 2010	114	200	77	197	3.4%	2.07 [1.38, 3.08]
Maha S. Al-Shammari 2017	266	660	405	1032	8.3%	1.05 [0.86, 1.28]
Riyadh Saif-Ali 2011	207	600	124	460	6.0%	1.43 [1.09, 1.86]
Vasiuddin Khan 2020	264	600	64	200	4.4%	1.67 [1.19, 2.34]
Wassim Y. Almawi 2013	845	1990	815	2152	11.7%	1.21 [1.07, 1.37]
Xing-Ping Dai 2012	298	734	143	428	6.5%	1.36 [1.06, 1.75]
Y. Liu 2009	1304	3770	1221	3988	13.1%	1.20 [1.09, 1.32]
Yiping Li 2020	802	2388	790	2584	11.9%	1.15 [1.02, 1.29]
Total (95% CI)		**22566**		**23311**	**100.0%**	**1.19 [1.09, 1.29]**
Total events	8899		8542			

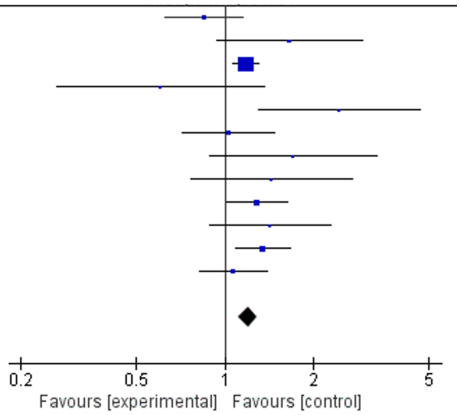
Heterogeneity: Tau² = 0.01; Chi² = 33.54, df = 11 (P = 0.0004); I² = 67%
Test for overall effect: Z = 4.09 (P < 0.0001)



B

Study or subgroup	T2DM Events	T2DM Total	Control Events	Control Total	Weight	Odds ratio M-H, fixed, 95%CI
Amira Turki 2012	107	886	80	574	6.7%	0.85 [0.62, 1.16]
Fulan Hu 2021	33	277	21	279	1.5%	1.66 [0.94, 2.95]
Jana V. van Vliet-Ostaptchouk 2012	869	4549	863	5182	51.4%	1.18 [1.07, 1.31]
L.J. Cui 2016	11	100	17	100	1.2%	0.60 [0.27, 1.36]
Li Zhang 2010	39	100	20	97	1.0%	2.46 [1.30, 4.65]
Maha S. Al-Shammari 2017	58	320	91	516	4.5%	1.03 [0.72, 1.49]
Riyadh Saif-Ali 2011	30	300	14	230	1.1%	1.71 [0.89, 3.31]
Vasiuddin Khan 2020	57	300	14	100	1.3%	1.44 [0.76, 2.72]
Wassim Y. Almawi 2013	174	995	152	1076	9.5%	1.29 [1.02, 1.63]
Xing-Ping Dai 2012	65	367	28	214	2.3%	1.43 [0.89, 2.31]
Y. Liu 2009	209	1885	169	1994	11.5%	1.35 [1.09, 1.67]
Yiping Li 2020	117	1194	119	1292	8.1%	1.07 [0.82, 1.40]
Total (95% CI)		**11273**		**11654**	**100.0%**	**1.20 [1.11, 1.29]**
Total events	1769		1588			

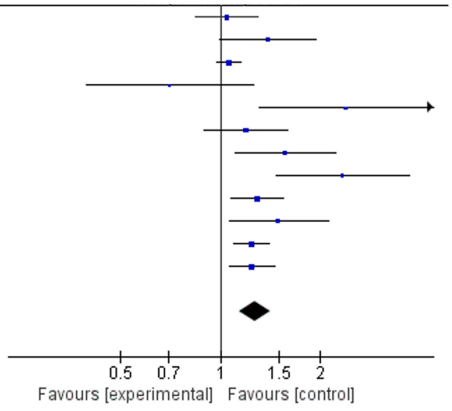
Heterogeneity: Chi² = 18.51, df = 11 (P = 0.07); I² = 41%
Test for overall effect: Z = 4.82 (P < 0.00001)



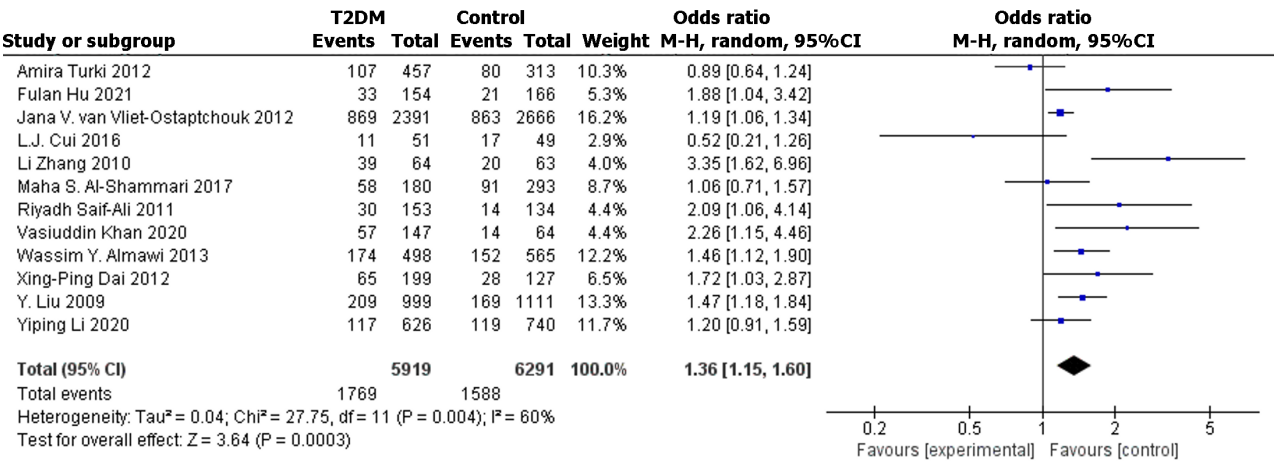
C

Study or subgroup	T2DM Events	T2DM Total	Control Events	Control Total	Weight	Odds ratio M-H, random, 95%CI
Amira Turki 2012	536	886	341	574	10.2%	1.05 [0.84, 1.30]
Fulan Hu 2021	156	277	134	279	6.7%	1.40 [1.00, 1.95]
Jana V. van Vliet-Ostaptchouk 2012	3027	4549	3379	5182	15.0%	1.06 [0.98, 1.15]
L.J. Cui 2016	60	100	68	100	3.0%	0.71 [0.40, 1.26]
Li Zhang 2010	75	100	54	97	2.8%	2.39 [1.31, 4.37]
Maha S. Al-Shammari 2017	208	320	314	516	7.8%	1.19 [0.89, 1.60]
Riyadh Saif-Ali 2011	177	300	110	230	6.4%	1.57 [1.11, 2.22]
Vasiuddin Khan 2020	210	300	50	100	4.3%	2.33 [1.47, 3.71]
Wassim Y. Almawi 2013	671	995	663	1076	11.5%	1.29 [1.08, 1.55]
Xing-Ping Dai 2012	233	367	115	214	6.5%	1.50 [1.06, 2.11]
Y. Liu 2009	1095	1885	1052	1994	13.5%	1.24 [1.09, 1.41]
Yiping Li 2020	685	1194	671	1292	12.3%	1.25 [1.06, 1.46]
Total (95% CI)		**11273**		**11654**	**100.0%**	**1.27 [1.14, 1.42]**
Total events	7133		6951			

Heterogeneity: Tau² = 0.02; Chi² = 31.32, df = 11 (P = 0.0010); I² = 65%
Test for overall effect: Z = 4.21 (P < 0.0001)



D



E

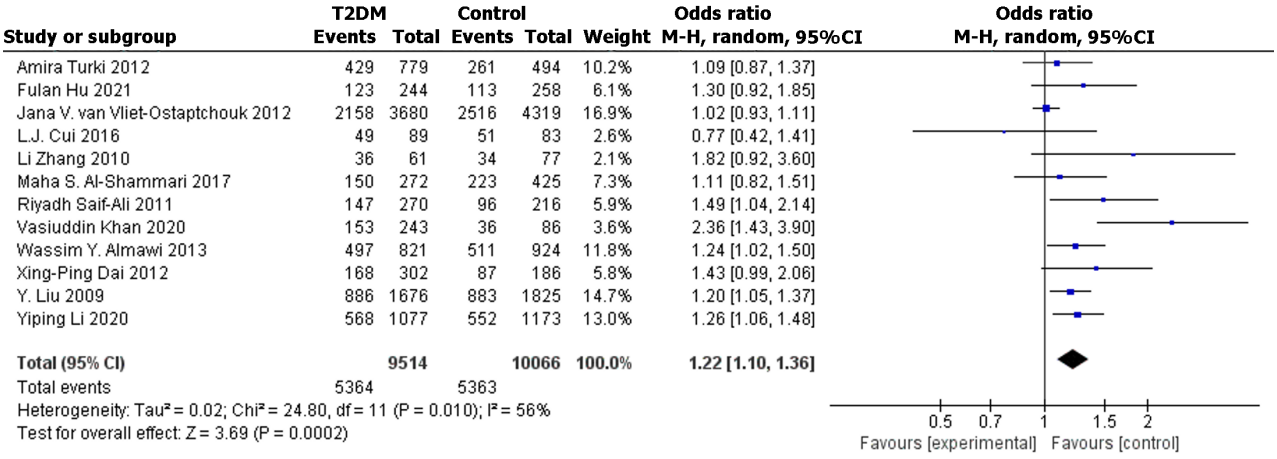


Figure 2 The forest plot of different model. A: Allelic model; B: Recessive model; C: Dominant model; D: Co-dominant model (CC *vs* AA); E: Co-dominant model (AC *vs* AA). T2DM: Type 2 diabetes mellitus.

DISCUSSION

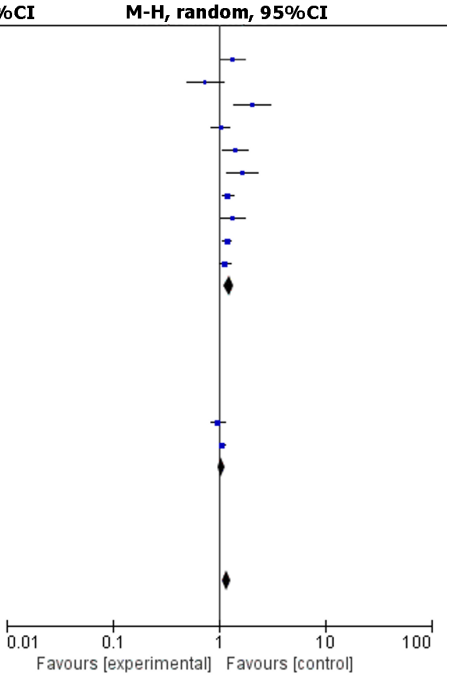
Compared with previous studies, we increased the inclusion criteria of cases, excluded the interference of other factors (*e.g.*, gestational diabetes), improved the strength of proof of the study, and made the results more reliable and stable. Our meta-analysis supported the findings of Khan *et al*[10], suggesting that the rs2237895 SNP in the *KCNQ1* gene is significantly associated with the development of T2DM in Asian populations. In the study by Cui *et al*[7], the study population had an overall overweight problem, which increased the risk of T2DM prevalence and thus confounded the findings[22].

T2DM is a multifactorial, chronic, metabolic disease[23]. The idea that genetic factors have a significant role in the development of T2DM is now more widely accepted[23], although only a few genes have been confirmed as a risk for the development of T2DM. However, many genetic characteristics associated with T2DM, such as effect sizes and risk allele frequencies, need to be explored[24]. There is a need for researchers to identify risk genetic loci for T2DM and characterize the variation at the loci, thus providing a basis for elucidating the genetic pathogenesis of T2DM.

Previous studies have shown that the *KCNQ1*, *miR-21*, and *Arg972* may be risk genes for T2DM[25,26]. *KCNQ1* gene has now been shown to be located on chromosome 11p15.5, which is approximately 400 kb in length and consists of 17 exons ranging from 47 to 1122 bp in length[27]. *KCNQ1* is associated with voltage-gated K^+ channels, and mutations in the *KCNQ1* gene lead to dysfunction of K^+ channels, which would cause diseases such as QT syndrome and familial atrial fibrillation. *KCNQ1* is expressed in many tissues[27,28], and the more studied about the *KCNQ1* gene is expressed in cardiac and pancreatic tissues[29]. Current studies suggest that the main mechanisms of T2DM development are insulin resistance and islet β-cell dysfunction[2,23]. Variants in the *KCNQ1* gene may lead to increased susceptibility to T2DM in the population by altering insulin secretion from pancreatic β-cells[30,31]. It was hypothesized that variants in the *KCNQ1* gene would lead to increased expression of *KCNQ1* protein on pancreatic β-cells, which in turn would alter the open state of voltage-gated potassium channels, decrease insulin secretion, and impair glucose storage and utilization[32].

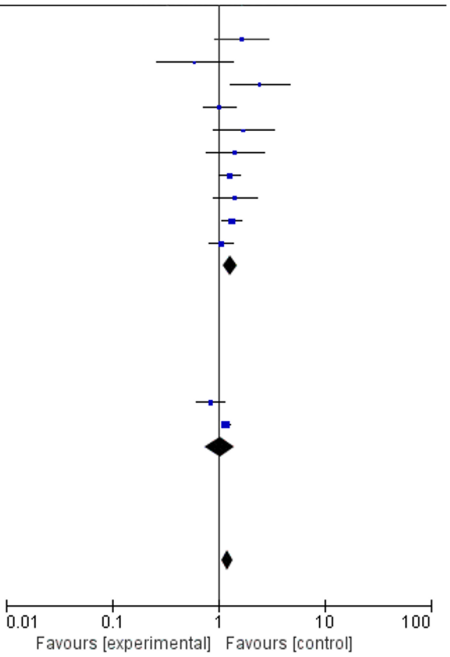
A

Study or subgroup	T2DM Events	T2DM Total	Control Events	Control Total	Weight	Odds ratio M-H, random, 95%CI
1.2.1 Asian						
Fulan Hu 2021	189	554	155	558	6.4%	1.35 [1.04, 1.74]
L.J. Cui 2016	71	200	85	200	3.4%	0.74 [0.50, 1.11]
Li Zhang 2010	114	200	77	197	3.4%	2.07 [1.38, 3.08]
Maha S. Al-Shammari 2017	266	660	405	1032	8.3%	1.05 [0.86, 1.28]
Riyadh Saif-Ali 2011	207	600	124	460	6.0%	1.43 [1.09, 1.86]
Vasiuddin Khan 2020	264	600	64	200	4.4%	1.67 [1.19, 2.34]
Wassim Y. Almawi 2013	845	1990	815	2152	11.7%	1.21 [1.07, 1.37]
Xing-Ping Dai 2012	298	734	143	428	6.5%	1.36 [1.06, 1.75]
Y. Liu 2009	1304	3770	1221	3988	13.1%	1.20 [1.09, 1.32]
Yiping Li 2020	802	2388	790	2584	11.9%	1.15 [1.02, 1.29]
Subtotal (95% CI)		**11696**		**11799**	**75.1%**	**1.25 [1.13, 1.37]**
Total events	4360		3879			
Heterogeneity: Tau² = 0.01; Chi² = 21.78, df = 9 (P = 0.010); I² = 59%						
Test for overall effect: Z = 4.38 (P < 0.0001)						
1.2.2 Non-Asian						
Amira Turki 2012	643	1772	421	1148	10.2%	0.98 [0.84, 1.15]
Jana V. van Vliet-Ostaptchouk 2012	3896	9098	4242	10364	14.7%	1.08 [1.02, 1.14]
Subtotal (95% CI)		**10870**		**11512**	**24.9%**	**1.06 [0.98, 1.14]**
Total events	4539		4663			
Heterogeneity: Tau² = 0.00; Chi² = 1.27, df = 1 (P = 0.26); I² = 21%						
Test for overall effect: Z = 1.56 (P = 0.12)						
Total (95% CI)		**22566**		**23311**	**100.0%**	**1.19 [1.09, 1.29]**
Total events	8899		8542			
Heterogeneity: Tau² = 0.01; Chi² = 33.54, df = 11 (P = 0.0004); I² = 67%						
Test for overall effect: Z = 4.09 (P < 0.0001)						
Test for subgroup differences: Chi² = 6.55, df = 1 (P = 0.01), I² = 84.7%						

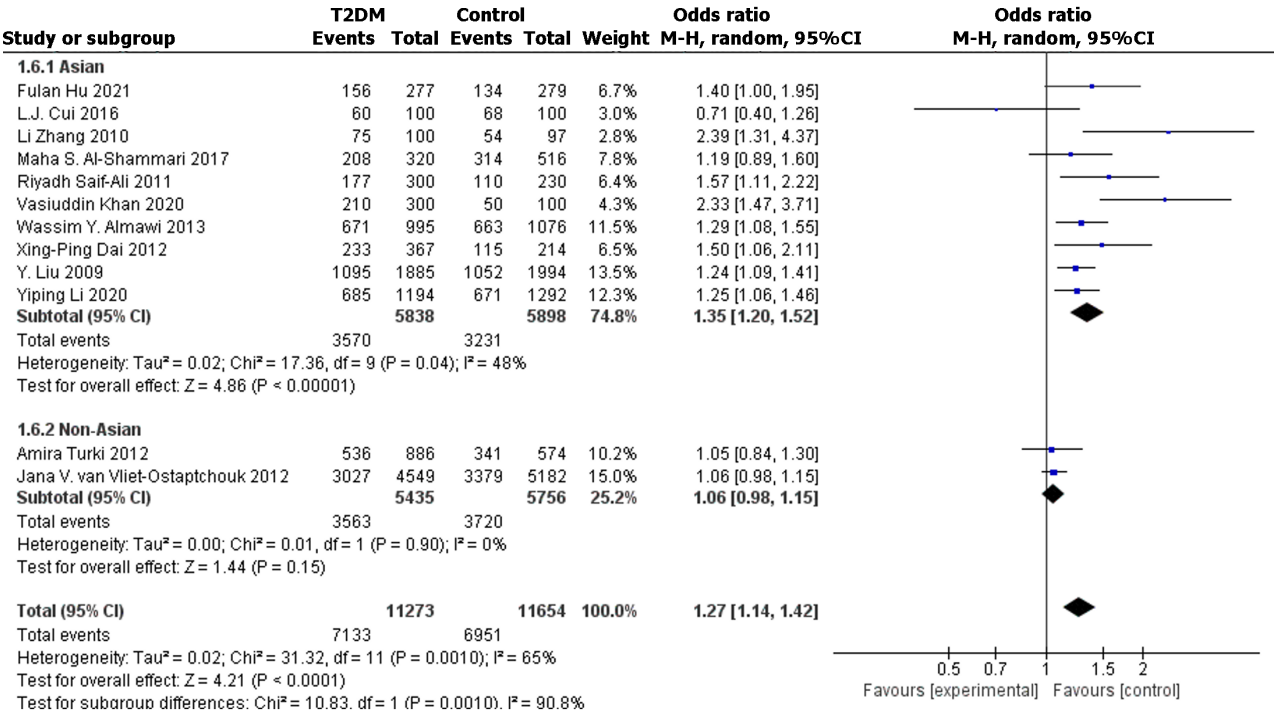


B

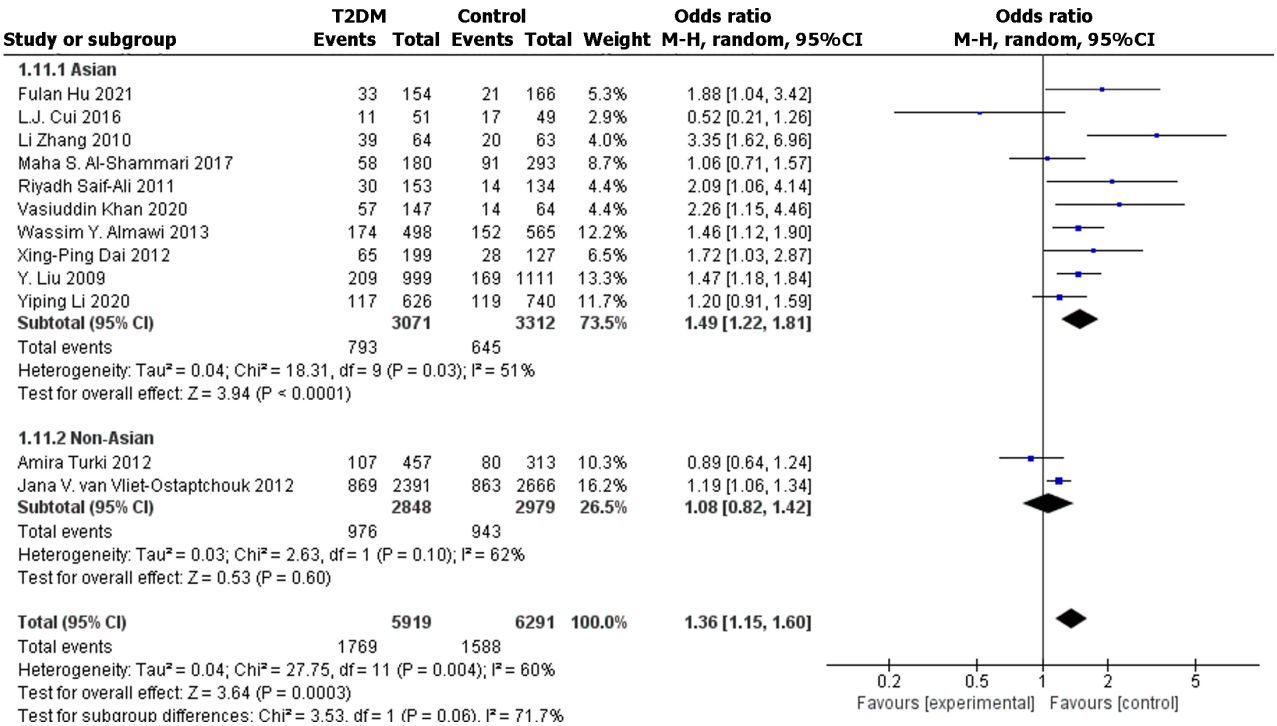
Study or subgroup	T2DM Events	T2DM Total	Control Events	Control Total	Weight	Odds ratio M-H, random, 95%CI
1.4.1 Asian						
Fulan Hu 2021	33	277	21	279	3.9%	1.66 [0.94, 2.95]
L.J. Cui 2016	11	100	17	100	2.1%	0.60 [0.27, 1.36]
Li Zhang 2010	39	100	20	97	3.3%	2.46 [1.30, 4.65]
Maha S. Al-Shammari 2017	58	320	91	516	8.0%	1.03 [0.72, 1.49]
Riyadh Saif-Ali 2011	30	300	14	230	3.1%	1.71 [0.89, 3.31]
Vasiuddin Khan 2020	57	300	14	100	3.3%	1.44 [0.76, 2.72]
Wassim Y. Almawi 2013	174	995	152	1076	13.2%	1.29 [1.02, 1.63]
Xing-Ping Dai 2012	65	367	28	214	5.3%	1.43 [0.89, 2.31]
Y. Liu 2009	209	1885	169	1994	14.6%	1.35 [1.09, 1.67]
Yiping Li 2020	117	1194	119	1292	11.6%	1.07 [0.82, 1.40]
Subtotal (95% CI)		**5838**		**5898**	**68.5%**	**1.29 [1.11, 1.49]**
Total events	793		645			
Heterogeneity: Tau² = 0.01; Chi² = 12.47, df = 9 (P = 0.19); I² = 28%						
Test for overall effect: Z = 3.38 (P = 0.0007)						
1.4.2 Non-Asian						
Amira Turki 2012	107	886	80	574	9.8%	0.85 [0.62, 1.16]
Jana V. van Vliet-Ostaptchouk 2012	869	4549	863	5182	21.7%	1.18 [1.07, 1.31]
Subtotal (95% CI)		**5435**		**5756**	**31.5%**	**1.04 [0.75, 1.42]**
Total events	976		943			
Heterogeneity: Tau² = 0.04; Chi² = 3.93, df = 1 (P = 0.05); I² = 75%						
Test for overall effect: Z = 0.21 (P = 0.83)						
Total (95% CI)		**11273**		**11654**	**100.0%**	**1.21 [1.07, 1.37]**
Total events	1769		1588			
Heterogeneity: Tau² = 0.02; Chi² = 18.51, df = 11 (P = 0.07); I² = 41%						
Test for overall effect: Z = 3.08 (P = 0.002)						
Test for subgroup differences: Chi² = 1.50, df = 1 (P = 0.22), I² = 33.2%						



C


T2DM
Control
Odds ratio
Odds ratio
Study or subgroup Events Total Events Total Weight M-H, random, 95%CI
M-H, random, 95%CI
1.6.1 Asian
Fulan Hu 2021 156 277 134 279 6.7% 1.40 [1.00, 1.95]
L.J. Cui 2016 60 100 68 100 3.0% 0.71 [0.40, 1.26]
Li Zhang 2010 75 100 54 97 2.8% 2.39 [1.31, 4.37]
Maha S. Al-Shammari 2017 208 320 314 516 7.8% 1.19 [0.89, 1.60]
Riyadh Saif-Ali 2011 177 300 110 230 6.4% 1.57 [1.11, 2.22]
Vasiuddin Khan 2020 210 300 50 100 4.3% 2.33 [1.47, 3.71]
Wassim Y. Almawi 2013 671 995 663 1076 11.5% 1.29 [1.08, 1.55]
Xing-Ping Dai 2012 233 367 115 214 6.5% 1.50 [1.06, 2.11]
Y. Liu 2009 1095 1885 1052 1994 13.5% 1.24 [1.09, 1.41]
Yiping Li 2020 685 1194 671 1292 12.3% 1.25 [1.06, 1.46]
Subtotal (95% CI) 5838 5898 74.8% 1.35 [1.20, 1.52]
Total events 3570 3231
Heterogeneity: Tau² = 0.02; Chi² = 17.36, df = 9 (P = 0.04); I² = 48%
Test for overall effect: Z = 4.86 (P < 0.00001)
1.6.2 Non-Asian
Amira Turki 2012 536 886 341 574 10.2% 1.05 [0.84, 1.30]
Jana V. van Vliet-Ostaptchouk 2012 3027 4549 3379 5182 15.0% 1.06 [0.98, 1.15]
Subtotal (95% CI) 5435 5756 25.2% 1.06 [0.98, 1.15]
Total events 3563 3720
Heterogeneity: Tau² = 0.00; Chi² = 0.01, df = 1 (P = 0.90); I² = 0%
Test for overall effect: Z = 1.44 (P = 0.15)
Total (95% CI) 11273 11654 100.0% 1.27 [1.14, 1.42]
Total events 7133 6951
Heterogeneity: Tau² = 0.02; Chi² = 31.32, df = 11 (P = 0.0010); I² = 65%
Test for overall effect: Z = 4.21 (P < 0.0001)
Test for subgroup differences: Chi² = 10.83, df = 1 (P = 0.0010), I² = 90.8%
0.5 0.7 1 1.5 2
Favours [experimental] Favours [control]

D


T2DM
Control
Odds ratio
Odds ratio
Study or subgroup Events Total Events Total Weight M-H, random, 95%CI
M-H, random, 95%CI
1.11.1 Asian
Fulan Hu 2021 33 154 21 166 5.3% 1.88 [1.04, 3.42]
L.J. Cui 2016 11 51 17 49 2.9% 0.52 [0.21, 1.26]
Li Zhang 2010 39 64 20 63 4.0% 3.35 [1.62, 6.96]
Maha S. Al-Shammari 2017 58 180 91 293 8.7% 1.06 [0.71, 1.57]
Riyadh Saif-Ali 2011 30 153 14 134 4.4% 2.09 [1.06, 4.14]
Vasiuddin Khan 2020 57 147 14 64 4.4% 2.26 [1.15, 4.46]
Wassim Y. Almawi 2013 174 498 152 565 12.2% 1.46 [1.12, 1.90]
Xing-Ping Dai 2012 65 199 28 127 6.5% 1.72 [1.03, 2.87]
Y. Liu 2009 209 999 169 1111 13.3% 1.47 [1.18, 1.84]
Yiping Li 2020 117 626 119 740 11.7% 1.20 [0.91, 1.59]
Subtotal (95% CI) 3071 3312 73.5% 1.49 [1.22, 1.81]
Total events 793 645
Heterogeneity: Tau² = 0.04; Chi² = 18.31, df = 9 (P = 0.03); I² = 51%
Test for overall effect: Z = 3.94 (P < 0.0001)
1.11.2 Non-Asian
Amira Turki 2012 107 457 80 313 10.3% 0.89 [0.64, 1.24]
Jana V. van Vliet-Ostaptchouk 2012 869 2391 863 2666 16.2% 1.19 [1.06, 1.34]
Subtotal (95% CI) 2848 2979 26.5% 1.08 [0.82, 1.42]
Total events 976 943
Heterogeneity: Tau² = 0.03; Chi² = 2.63, df = 1 (P = 0.10); I² = 62%
Test for overall effect: Z = 0.53 (P = 0.60)
Total (95% CI) 5919 6291 100.0% 1.36 [1.15, 1.60]
Total events 1769 1588
Heterogeneity: Tau² = 0.04; Chi² = 27.75, df = 11 (P = 0.004); I² = 60%
Test for overall effect: Z = 3.64 (P = 0.0003)
Test for subgroup differences: Chi² = 3.53, df = 1 (P = 0.06), I² = 71.7%
0.2 0.5 1 2 5
Favours [experimental] Favours [control]

E

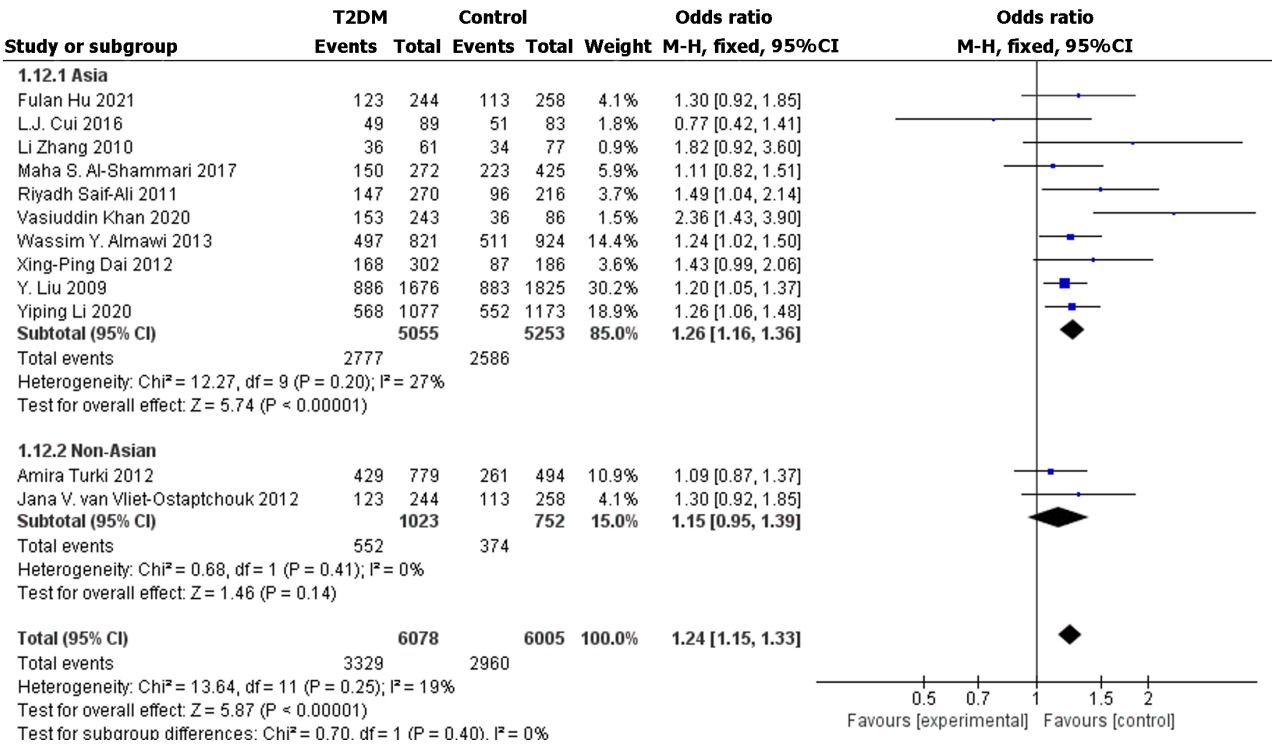


Figure 3 The forest plot for stratified analysis of different model. A: Allelic model; B: Recessive model; C: Dominant model; D: Co-dominant model (CC *vs* AA); E: Co-dominant model (AC *vs* AA). T2DM: Type 2 diabetes mellitus.

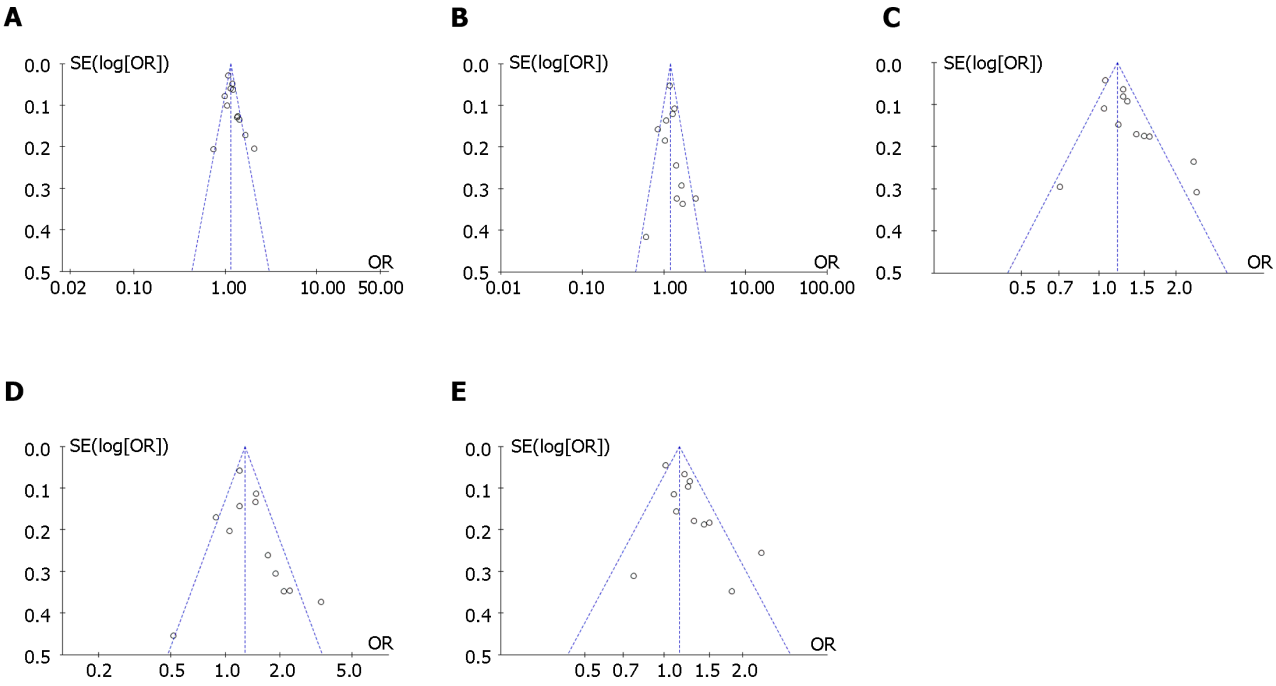


Figure 4 The funnel plot of different model. A: Allelic model; B: Recessive model; C: Dominant model; D: Co-dominant model (CC *vs* AA); E: Co-dominant model (AC *vs* AA). OR: Odds ratio.

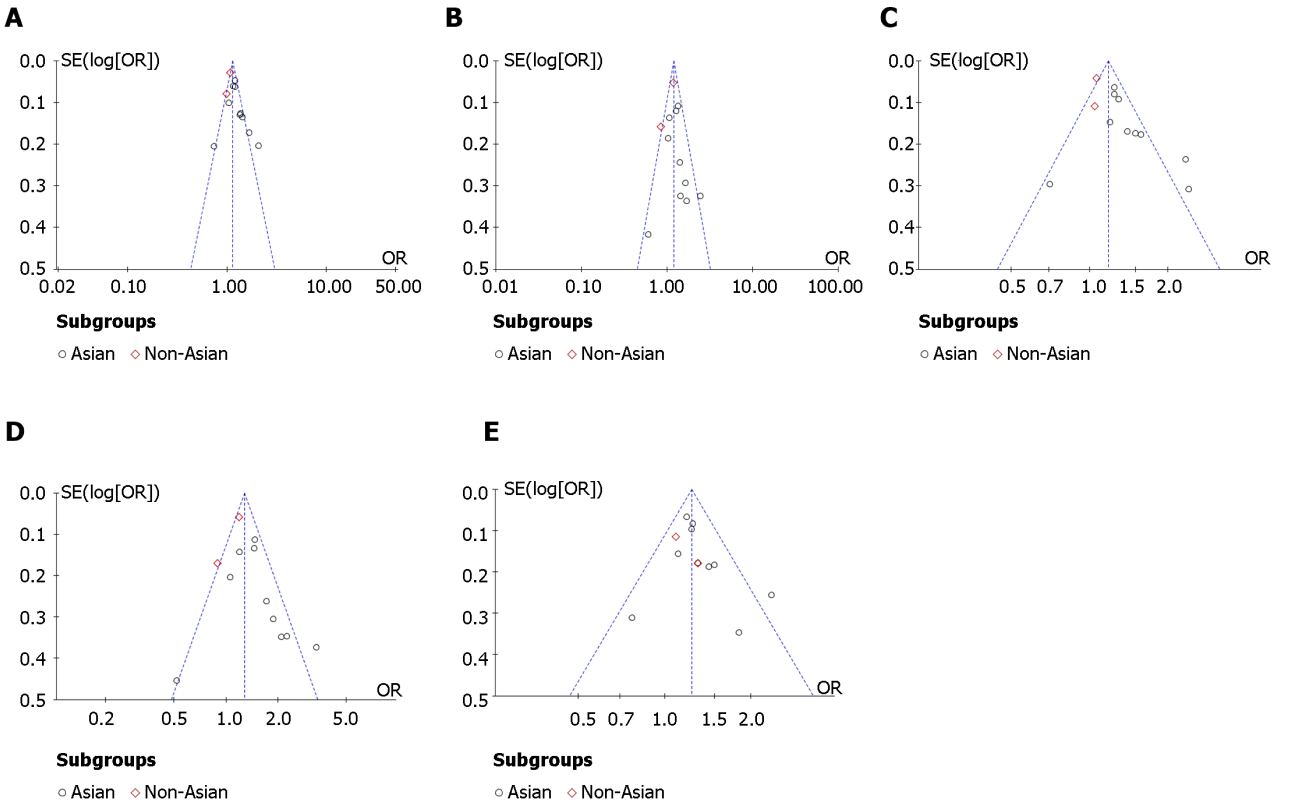


Figure 5 The funnel plot for stratified analysis of different models. A: Allelic model; B: Recessive model; C: Dominant model; D: Co-dominant model (CC *vs* AA); E: Co-dominant model (AC *vs* AA). OR: Odds ratio.

This meta-analysis of the *KCNQ1* gene rs2237895 SNP and T2DM association study involved 12 studies, including 11273 T2DM patients and 11654 controls. This analysis showed that the rs2237895 polymorphism was significantly associated with an elevated risk of developing T2DM in an Asian population, which is consistent with Khan *et al*'s[10] findings. In Asian populations, C allele carriers have an increased risk of developing T2DM. The risk of T2DM is also increased in people with the CC and AC genotypes compared to the AA genotype. This is consistent with the previous findings of Hu *et al*[16]. Also, their findings showed that rs2237895 was associated with hypertension, body mass index, and hypertriglyceridemia. In non-Asian populations, this association was not significant. A 2015 study by Ríos *et al*[33] in Europeans also showed that the *KCNQ1* gene rs2237895 SNP was not significantly associated with T2DM, which is consistent with our findings. Our work provided strong evidence for the genetic pathogenesis of T2DM and helped to fully reveal the pathogenesis of T2DM.

This study showed that the rs2237895 SNP of the *KCNQ1* gene was differentially associated with T2DM in different populations. The reasons for this variation may be mutations in the regulatory region of the *KCNQ1* gene in particular populations[33], which interfere with the expression of the *KCNQ1* gene; or it may be due to the existence of different genotypes and allele frequencies in populations with different clinical characteristics, geographical distribution and ethnic origin; or differences in the external influences associated with T2DM, such as lifestyle and behavior, in different populations[4,23-25]. The possibility of false-negative results in non-Asian populations with small study sample sizes cannot be excluded.

CONCLUSION

In the Asian population, there was a significant association between the *KCNQ1* gene rs2237895 SNP and T2DM onset. C allele carriers were at increased risk of T2DM, and the CC and AC genotypes significantly increased the susceptibility to T2DM. However, in the non-Asian population, the association between rs2237895 and T2DM onset was not significant.

ARTICLE HIGHLIGHTS

Research background

The association between the rs2237895 single nucleotide polymorphism (SNP) in the *KCNQ1* gene and the prevalence of type 2 diabetes mellitus (T2DM) has been controversial in different studies.

Research motivation

The aim of this study was to investigate the association between the *KCNQ1* gene rs2237895 and the prevalence of T2DM, and to provide help in establishing the pathogenesis of T2DM.

Research objectives

Demonstration of the association of the rs2237895 SNP in the *KCNQ1* gene with the prevalence of T2DM. Also, to explore whether this relationship differs in different populations.

Research methods

We searched nine databases. Two authors independently screened the literature according to the established inclusion and exclusion criteria. Finally, data extraction was performed and the data were meta-analyzed.

Research results

Twelve case–control studies met our inclusion criteria. After analysis, the rs2237895 SNP in the *KCNQ1* gene was associated with T2DM prevalence in Asian populations. However, this association was not significant in non-Asian populations.

Research conclusions

In Asian populations, carriers of the rs2237895 C allele of the *KCNQ1* gene were highly susceptible to T2DM compared to those who did not carry the C allele. However, in non-Asian populations, the association between the rs2237895 SNP and T2DM was not significant.

Research perspectives

We should continue to search for T2DM susceptibility genes through advanced technologies (*e.g.*, genome-wide association strategy) and gradually elucidate the pathogenesis of T2DM.

FOOTNOTES

Co-first authors: Dong-Xu Li and Li-Ping Yin.

Co-corresponding authors: Chen-Sen He and Jiang-Jie Sun.

Author contributions: Li DX, Yin LP, Sun JJ, and He CS designed this study (substantial contributions to the conception); Li DX, Yin LP, Song YQ, Shao NN, and Zhu H collected data; Li DX and Yin LP extracted and analyzed data, interpretation of data for the work; Sun JJ and He CS provided guidance for statistical analysis and provided financial support. They agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy; Li DX and Yin LP wrote the manuscript; Li DX, Yin LP, Song YQ, Shao NN, Zhu H, Sun JJ and He CS reviewed the manuscript; Li DX and Yin LP contributed equally to this work as co-first authors; Sun JJ and He CS contributed equally to this work as co-corresponding authors. The reasons for designating Li DX and Yin LP as co-first authors are as follows. First, Li DX and Yin LP contributed equal effort throughout the study. The selection of these researchers as co-first authors respects their equal contributions. Second, the research was conducted as a collaborative effort, and the designation of co-first authors accurately reflects the distribution of responsibilities and burdens associated with the time and effort required to complete the research and final paper. The reasons for designating Sun JJ and He CS as co-corresponding authors are as follows. First, Sun JJ and He CS put equal effort into the entire study. Second, the designation of co-corresponding authors best reflects the need for this study to have authors from different fields, which promotes the most in-depth examination of the research topic. In summary, we believe that the designation of Li DX and Yin LP as co-first authors and Sun JJ and He CS as co-corresponding authors meets the requirements of our manuscript, which reflects the spirit of equality and cooperation in our team.

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LETTER TO THE EDITOR

Chiglitazar and Thiazolidinedione in patients with type 2 diabetes: Which is better?

Kotha Sugunakar Reddy, Archana Gaur, Sakthivadivel Varatharajan, Arvind Kumar Morya

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Abstract

This published Meta-Analysis by Lin *et al* is an indirect comparison between two drugs Chiglitazar and Thiazolidinedione which are commonly used for glycemic control in type-II diabetes mellitus. In terms of safety and efficacy, this Meta-Analysis is inconclusive.

Key Words: Type-II diabetes mellitus; Glucose intolerance; Hyperglycemia; Research methodology

Core Tip: The authors had done an indirect comparison between the new anti-diabetic drug Chiglitazar with Thiazolidinediones. It is premature to compare a single, relatively smaller study to 142 studies on Thiazolidinediones which are spanning over 28 years. Also, the efficacy of different thiazolidinediones has not been comprehensively compared and emphasized in the analysis and discussion.

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TO THE EDITOR

We have read with great interest the article entitled "Indirect Comparison of Efficacy and Safety of Chiglitazar and Thiazolidinedione in Patients with Type 2 Diabetes: A Meta-Analysis" authored by Lin C *et al*, published in the *World Journal of Diabetes* [2023; 14 (10): 1573-1584][1]. I would like to extend my sincere congratulations to the authors for conducting this comparative meta-analysis and contributing to the growing body of knowledge on oral hypoglycemic drugs.

With a diabetes pandemic in visibility, there is an urgent need of newer molecules and modality of treatments for type 2 diabetes mellitus, Chiglitazar represents a new wave of non- thiazolidinedione medications that can regulate gene expression by binding in a configuration-restricted manner and inhibiting the phosphorylation of hPPARγ, Chiglitazar operates as a pan-agonist, offering a detailed mechanism that elucidates its ability to fully activate PPARγ and partially activate PPARα and PPARβ[2].

The article under discussion offers a unique perspective by comparing the efficacy and safety of the newer molecule, Chiglitazar with the much older thiazolidinediones through an indirect meta-analysis. While this approach is commendable, it is important to acknowledge that adjusted indirect comparisons are not without their limitations, and they are subject to potential heterogeneity among the studies being compared[3]. Moreover, this method relies on a bridge comparator, which in this case, is the placebo used in the included studies.

It is worth noting that the article does not explicitly mention the specific method and type of indirect comparison used. However, it can be inferred that an adjusted indirect comparison with the Bucher Method was employed to estimate the relative effects of the two treatments[4]. One of the drawbacks of this method is that it assumes a similarity between the studies, which may not always hold true, especially given the potential heterogeneity among study populations, such as differences in races.

The comparison made in this article involves 142 studies on different thiazolidinediones, conducted over a 28-year span, compared to a single study conducted on 166 patients with Chiglitazar. This discrepancy in the quantity and timing of the studies is a critical factor to consider when drawing conclusions about the efficacy and safety of Chiglitazar in comparison to thiazolidinediones. The substantial time gap between the studies could result in variations in treatment guidelines, diagnostic criteria, and patient populations, which can influence the comparability of the results.

The present article on indirect meta-analysis discusses both the standard (32 mg) and augmented (48 mg) doses of Chiglitazar. However, it is notable that the article predominantly emphasizes the results related to the augmented dose's effects and safety without providing a comprehensive analysis of the standard dose results. Additionally, the rationale for using an augmented dose and the motivation for testing Chiglitazar with this dose are not sufficiently addressed. A randomized double-blind trial, conducted over 24 wk in a small group in China, explored the efficacy and changes in insulin resistance and retinol binding protein levels revealed no significant reductions in HbA1c levels from the baseline in the full analysis population for Chiglitazar at doses of 32 mg, 48 mg, and sitagliptin at 100 mg but Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) values in the Chiglitazar at 48 mg group were notably lower, HOMA-β levels for both Chiglitazar doses (32 mg and 48 mg) decreased significantly compared to the sitagliptin 100 mg group. Chiglitazar, at both doses, notably elevated total cholesterol and high-density lipoprotein cholesterol (HDL-C) compared to sitagliptin 100 mg[5].

When scrutinizing the statistical analysis and discussion, it becomes apparent that the study results for different thiazolidinediones and comparisons between them, such as Pioglitazone, Rosiglitazone, Troglitazone, and Englitazone's efficacy, are not adequately addressed. The forest plot displaying pooled efficacy from different thiazolidinedione study groups reveals considerable heterogeneity in efficacy endpoints, including HbA1c, low-density lipoprotein cholesterol, TG-C, HDL-C, FBS, HOMA-IR, and HOMA-Beta, with percentages ranging from 98% to 100%. Such high levels of heterogeneity can be considered problematic, and comparing pooled efficacy can be misleading. It would be more appropriate to explore alternative methods, such as matched adjusted indirect comparison, while including individual patient data to improve the accuracy of the analysis.

The collective indirect comparisons pertaining to safety endpoints, which encompass hypoglycemia, edema, bone fractures, upper respiratory tract infections, and urinary tract infections, do not exhibit statistically significant results. The confidence intervals for these comparisons are notably wide, indicating a lack of statistical significance.

In light of these limitations, it would be premature to draw confident conclusions regarding the preferability of Chiglitazar over thiazolidinediones, particularly when comparing a single, relatively smaller study to 142 studies spanning over 28 years. The efficacy of different thiazolidinediones has not been comprehensively compared and emphasized in the analysis and discussion. Therefore, the need for a more robust and nuanced evaluation remains.

In conclusion, I/we wish to express our gratitude to the authors for sharing their knowledge and research work, which involves comparing a newer molecule with older ones concerning efficacy and safety. This article serves as a source of motivation for healthcare professionals to delve deeper into the study of newer molecules like Chiglitazar, ultimately enriching the arsenal of oral hypoglycemic drugs and instilling growing confidence in our practice.

FOOTNOTES

Author contributions: Morya AK designed and formulated the research; Reddy KS, Gaur A and Varatharajan S performed the research; Reddy KS, Gaur A and Varatharajan S analyzed data and wrote the letter; and Morya AK revised the letter; all the authors have read and approved the final manuscript.

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LETTER TO THE EDITOR

Effects of vitamin family members on insulin resistance and diabetes complications

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Abstract

The following letter to the editor highlights the article "Effects of vitamin D supplementation on glucose and lipid metabolism in patients with type 2 diabetes mellitus and risk factors for insulin resistance" in *World J Diabetes* 2023 Oct 15; 14 (10): 1514-1523. It is necessary to explore the role of vitamin family members in insulin resistance and diabetes complications.

Key Words: Vitamin; Insulin resistance; Diabetes complications; Letter

Core Tip: Vitamins are a class of small molecular organic compounds that cannot be synthesized or are synthesized in extremely small amounts by the body. Many recent studies have shown that vitamin supplementation plays an important role in inhibiting inflammation, controlling blood sugar, and promoting insulin secretion in diabetic patients. However, the function of vitamin for diabetes remains to be studied. It is clinically significant to explore the effects of other vitamin family members on insulin resistance and diabetes complications.

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TO THE EDITOR

We read the article by Sun *et al*[1] entitled "Effects of vitamin D supplementation on glucose and lipid metabolism in patients with type 2 diabetes mellitus and risk factors for insulin resistance". The authors found that 25 hydroxyvitamin D3 [25(OH)D3], a vitamin D deficiency marker, is an independent risk factor for insulin resistance (IR) and promotes glucose metabolism. Many recent studies have shown that vitamin D supplementation plays an important role in inhibiting inflammation, controlling blood sugar, and promoting insulin secretion in diabetic patients[2,3]. Therefore, it is clinically significant to explore the effects of other vitamin family members on IR and diabetes complications.

With societal and economic development, the prevalence of diabetes continues to increase. Approximately 1.6 million deaths per year are attributed directly to diabetes[4]. Currently, diabetes is classified primarily as type 1 diabetes (T1D) and T2D. It is estimated that by 2030, 578 million people worldwide will be diagnosed with diabetes[5]; T2D is the most common diagnosis, accounting for approximately 90%. An increasing number of studies show that most T2D patients have IR[6]. IR is a weakening of the body's response to insulin, resulting in increased blood sugar levels; physiologically, IR is defined as a state of reduced responsiveness of insulin-targeted tissues to high insulin levels[7]. When IR begins to occur, insulin levels increase to meet normal insulin requirements; however, over time, these increased levels lead to hyperglycemia-induced islet β cell failure, chronic hyperinsulinemia, and eventually T2D.

Vitamins are a class of small molecular organic compounds that cannot be synthesized or are synthesized in extremely small amounts by the body. Vitamins are necessary to maintain the normal physiological functions of the human body, and they must be obtained from food. Primarily, vitamins fall into two categories: fat-soluble vitamins and water-soluble vitamins. Many recent studies have shown that vitamins are involved in regulating T2D *via* IR. In fact, vitamin K4 supplementation can improve IR in patients with T2D[8]. Vitamin K deficiency under β cell stress may lead to β cell dysfunction by reducing endoplasmic reticulum Gla protein (ERGP) gamma carboxylation, thus increasing the risk of T2D, particularly in the case of overnutrition. Gamma carboxylated ERGP is needed to prevent uncontrolled insulin secretion by β cells and maintain normal insulin secretion[9]. A recent review reported that vitamin E, especially alpha tocopherol, has been shown to reduce lipid peroxidation, and the superoxide produced by vitamin E can damage the β cell structure as well as vital functional components for maintaining normal glucose concentrations[10]. Another study has shown that deficiencies in folic acid, vitamin B6, and vitamin B12 can lead to dyslipidemia, vascular endothelial dysfunction, abnormal glucose tolerance, and oxidative stress, leading to IR[11]. Pramono *et al*[12] have shown that obesity and IR are usually related to vitamin D deficiency. Vitamin D directly stimulates insulin secretion by reducing pancreatic β cells through vitamin D receptor (VDR) and improves peripheral IR. Moreover, VDR deficiency in cardiovascular tissue increases ventricular fibroblast mass dysregulation and accelerates the myocardial fibrosis process[13]. Studies have also shown that serum vitamin D deficiency aggravates inflammation due to circulating gamma-delta T cells in T2D patients. 1α,25(OH)2D3/fructose-1,6-bisphosphatase 1 (FBP1) signal transduction can inhibit glycolysis in γδ T cells by promoting targeted FBP1 expression, thereby driving Akt/p38 MAPK dephosphorylation. This can also reduce inflammatory cytokine production (TNF-α and IFN-γ) in γδ T cells to alleviate IR. The role of vitamin D in IR regulation to alleviate diabetes may be mediated by stimulating insulin receptor expression, improving insulin levels, and regulating cytokine expression and the calcium pool in various tissues[14]. Upon examining the mechanism through which vitamin D affects IR, supplementation with 1,25(OH)2D3 combined with insulin was shown to significantly improve the expression of glucose transporter 4, a key protein in regulating glucose metabolism and maintaining glucose homeostasis through glucose uptake[15].

Further studies have found that vitamin D is involved in the pathological process of multiple diabetes complications. In diabetic cardiomyopathy, the 1,25-dihydroxyvitamin D receptor inhibits autophagy by inhibiting nuclear FOXO1 translocation, thereby alleviating diabetic heart damage[16]. Vitamin D also improves blood glucose and insulin levels, reduces NF-kB activity in heart tissue, and down-regulates advanced glycosylation end products and the hexosamine pathway to alleviate diabetic cardiomyopathy[17]. In diabetic kidney disease (DKD), vitamin D deficiency up-regulates zinc finger e-box binding homeobox and down-regulates miR-200b, which promotes the epithelial-mesenchymal transformation process and changes the renal structure and function of diabetic rats to accelerate DKD development[18]. Vitamin D supplementation can effectively reduce albuminuria and creatinine, markers of kidney disease in diabetic nephropathy patients[19]. 1,25(OH)2D3 also significantly inhibits the expression of sirtuin 1 (SIRT1) during oxidative stress[20], thereby attenuating renal oxidative damage. Increasing evidence has indicated that the vitamin D-VDR-RXR complex inhibits macrophage infiltration and immune effects in diabetic nephropathy[21]. Moreover, vitamin D deficiency is a potential risk factor for diabetic retinopathy (DR)[22]. Vitamin D is a strong antioxidant that can significantly reduce free radical formation, exert anti-inflammatory effects, and regulate autophagy and apoptosis; consequently, vitamin supplementation can help reduce the destructive effects of free radicals on DR[23]. Neuroprotective effects are exerted through the SIRT1/nrf-2/NF-kB signaling pathway[24]. 25(OH)D3 may inhibit oxidative stress in human retinal microvascular endothelial cells induced by high glucose-mediated miR-93 down-regulation[25]. Supplementation with vitamin D also improves neuropathy[26], which might be related to its regulation of neurotrophic factor levels and neuronal calcium homeostasis. Additional studies have shown that vitamin D deficiency has a greater effect on

the long-term chronic complications of diabetes, especially in patients with painful diabetic neuropathy, where vitamin D supplementation can effectively improve pain symptoms and nerve function in patients[27].

In addition, IR-induced glucose abnormalities and lipotoxicity, resulting in unbalanced fatty acid intake, are key factors in diabetic cardiomyopathy[28]. Abnormal coronary microcirculation, mitochondrial dysfunction, subcellular component abnormalities, and myocardial insulin signaling and calcium homeostasis impairments were observed in IR states. These pathophysiological changes can lead to diastolic dysfunction, fibrosis[29], and hypertrophy, ultimately causing heart failure[30]. Impaired insulin signaling is an important pathophysiological abnormality in diabetic cardiomyopathy. However, supplementation with vitamin D can significantly improve glucose tolerance and insulin sensitivity[31]. Vitamin D plays a key role in insulin receptor expression and increases glucose transporter function in insulin reactivity [13].

In summary, Sun *et al*[1] investigated the protective effects of vitamin D supplementation on IR in diabetic patients, but they did not include a pathological exploration of diabetic complications. The mechanism through which vitamin D acts in the treatment of IR and multiple diabetes complications remains to be studied.

FOOTNOTES

Author contributions: Chen HJ and Yang SY designed this letter; Chen HJ, Wang M, and Zou DM wrote this comment; Liang GY and Yang SY reviewed and supervised this manuscript; all authors approved the final version of the article.

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LETTER TO THE EDITOR

Regulatory role of peroxynitrite in advanced glycation end products mediated diabetic cardiovascular complications

Asis Bala

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Abstract

The Advanced Glycation End Products (AGE) binding with its receptor can increase reactive oxygen species (ROS) generation through specific signaling mediators. The effect of superoxide (O_2^-) and O_2^- mediated ROS and reactive nitrogen species depends on their concentration and location of formation. Nitric oxide (NO) has anti-inflammatory and anticoagulant properties and a vasodilation effect, but NO can be deactivated by reacting with O_2^-. This reaction between NO and O_2^- produces the potent oxidant $ONOO^-$. Therefore, ONOO-'s regulatory role in AGEs in diabetic cardiovascular complications must considered as a regulator of cardiovascular complications in diabetes.

Key Words: Diabetes; Cardiovascular complication; Advanced glycation end products; Reactive oxygen species; Reactive nitrogen species; Peroxynitrite

Core Tip: The binding of Advanced Glycation End Products (AGE) to its receptor triggers the activation of signaling mediators that promote the generation of reactive oxygen species (ROS). The impact of ROS on the body can be beneficial or harmful, depending on its concentration and location. In diabetic cardiovascular complications, peroxynitrite ($ONOO^-$) plays a crucial role in vascular changes. ROS, derived from NADPH oxidase, regulates host immune responses and cellular inflammation. The production of superoxide (O_2^-), hydrogen peroxide (H_2O_2), and other compounds occurs as oxygen undergoes a series of reductions. It is essential to consider the presence of $ONOO^-$ in AGEs in diabetic cardiovascular complications.

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TO THE EDITOR

I am writing to express my appreciation for the article published by Bansal *et al*[1] in the *World Journal of Diabetes* in 2023, titled "Advanced glycation end products: Key mediator and therapeutic target of cardiovascular complications in diabetes". The article provides a clear explanation of the role of Advanced Glycation End Products (AGE) in cardiovascular complications.

I want to draw attention to the role of superoxide (O_2^-) in connection to AGE, reactive oxygen species (ROS), and reactive nitrogen species (RNS) mediated immune inflammation. The article comprehensively outlined the impact of AGE on diabetic cardiovascular disease, encompassing both cellular and extracellular pathological effects. These effects include extracellular matrix oxidation, glycation of low-density lipoprotein, and the triggering of inflammatory signaling cascades, such as NADPH oxidase, NRF-2, NFκB, JAK, and STAT pathways. On the contrary, the article partially emphasized the significant role of Nitric oxide (NO) and NO synthase (NOS) in regulating AGE formation.

As mentioned in the article, AGE binding with its receptor increases ROS generation through stimulation of specific signaling mediators such as ERK, phospholipase A2, phosphoinositide 3-kinase activation, activation of NADPH oxidase, inducible NOS, PKC, and p38 MAPK[2]. However, the beneficial or detrimental role of O_2^- and O_2^- -mediated ROS or RNS is determined by its concentration and the places where it is formed[3]. Studies have shown that O_2^- immediately interacts with NO to produce the highly toxic peroxynitrite ($ONOO^-$), which plays a crucial role in vascular changes in diabetic cardiovascular complications[4,5].

The damage to vascular endothelial cells is a leading cause of diabetic vascular complications, which can be combated using endothelial progenitor cells (EPCs)[6]. The activation of various pathways such as xanthine and NAD(P)H oxidases, uncoupled NOS, cyclooxygenase, glucose autoxidation, the mitochondrial respiratory chain, polyol, and AGEs is triggered by hyperglycemia[4,7]. These pathways lead to the production of superoxide anion (O_2^-)[4,5]. The generation of superoxide due to hyperglycemia can also increase NO generation by enhancing the expression of NOSs by activating NF-κB[8]. However, O_2^- can quench NO, reducing the efficacy of the endothelium-derived vasodilator system[4]. Moreover, superoxide dismutase can convert superoxide to hydrogen peroxide (H_2O_2), which can react further with NO to form $ONOO^-$[9]. $ONOO^-$ can cause damage to cells by initiating lipid peroxidation, inactivating enzymes and proteins *via* oxidation and nitration, and activating matrix metalloproteinases[10]. Additionally, $ONOO^-$ can decrease the membrane potential by acting on mitochondria, triggering the release of proapoptotic factors such as cytochrome c and apoptosis-inducing factor[4,9,10]. These factors can mediate caspase-dependent and -independent apoptotic death pathways, which may contribute to the progression of diabetic cardiovascular complications[4]. Therefore, $ONOO^-$ is considered one of the critical modulators of diabetic cardiovascular complications since high glucose levels can impair EPC function and reduce NO production.

Furthermore, NADPH oxidase-derived ROS have become critical regulators of host immune responses and cellular inflammation[11,12]. Activation of phospholipase A2 in human neutrophils and other inflammatory cells by polyunsaturated fatty acids stimulates O_2^- production, triggering innate immune reactions. Increased O_2^- production may also activate the arachidonic acid pathways[5]. Oxygen undergoes a series of univalent reductions, sequentially producing O_2^-, H_2O_2, *etc*. NO always shows its anti-inflammatory, anticoagulant properties and vasodilation effect. Still, it can be inactivated by reaction with O_2^-, producing the potent oxidant $ONOO^-$[11-13]. Therefore, the regulatory role of $ONOO^-$ in AGEs in diabetic cardiovascular complications also needs to be considered.

FOOTNOTES

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