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ABOUT COVER

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AIMS AND SCOPE

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EDITORIAL

Nε-carboxymethyl-lysine and inflammatory cytokines, markers and mediators of coronary artery disease progression in diabetes

Sonia Eiras

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Abstract

This editorial refers to the article "Comparative analysis of Nε-carboxymethyl-lysine and inflammatory markers in diabetic and non-diabetic coronary artery disease patients", published in the recent issue of the *World Journal of Diabetes* 2023 is based on glucose metabolism, advanced glycation end products (AGEs), inflammation and adiposity on diabetes and coronary artery disease (CAD). This study has included CAD patients who were stratified according to glycosylated hemoglobin higher than 6.5 and sex-matched. A higher prevalence of hypertension, dyslipidemia, and non-vegetarian diet were found in the diabetic group. These risk factors might influence body weight and adiposity and explain the increment of the left atrium. Although this data was not supported by the study. The diet can also explain the non-enzymatic reactions on lipids, proteins, or nucleic acids and consequently an increment of AGEs. These molecules can emit fluorescence. However, one of the non-fluorescent and most abundant AGEs is Nε-carboxymethyl-lysine (CML). Its association with coronary artery stenosis and severity in the diabetic group might suggest its role as a player in CAD progression. Thus, CML, after binding with its receptor (RAGE), can induce calcification cascade through reactive oxygen species and mitogen-activated protein kinase. Moreover, this interaction AGE-RAGE can cause activation of the transcription nuclear factor-kb and induce inflammatory cytokines. It might explain the relationship between CML and pro-inflammatory cytokines in diabetic and CAD patients. Although this is a population from one center, the determination of CML and inflammatory cytokines might improve the diagnosis of severe and progressive CAD. Future and comparative studies among glycosylated hemoglobin, CML, and other AGE levels according to diagnosis and prognosis value might modify the clinical practice. Although these molecules are irreversible, they can act through a specific receptor inducing a signal transduction that might be modulated by inhibitors, antibodies, or siRNA. Further mechanistic studies might improve the development of future preventive therapies for diabetic patients.

Key Words: Nε-carboxymethyl-lysine; Inflammatory cytokines; Adiposity; Diabetes; Coronary artery disease

Core Tip: Coronary artery disease (CAD) is associated with 17.8 million deaths annually and nearly 30% have diabetes with insulin resistance. This metabolic disorder increases the circulating glucose levels that allow the non-enzymatic modifications of proteins, lipids, nucleic acids, *etc*. and form advanced glycation end products (AGEs). Glycosylated hemoglobin is considered a diagnostic marker for diabetes and a risk factor for CAD. However, AGEs through its receptor (RAGE) might increase signal transduction and consequently, inflammatory cytokines, and endothelial dysfunction and be markers and mediators of CAD.

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INTRODUCTION

Cardiovascular disease and obesity and type 2 diabetes mellitus

Cardiovascular disease (CVD) is the major cause of mortality and affects 32% of patients with type 2 diabetes mellitus (T2DM)[1]. This disorder is linked to obesity and a reduction of insulin signaling in cells[2]. Obesity is associated with an increment of stored energy on adipocytes that develop hypertrophy[3] and increase the inflammatory cells' attraction.

Dysfunctional epicardial fat

Computerized tomography (CT) of coronary arteries with suspected coronary artery disease (CAD) determined an accumulation of adipose tissue around them[4]. However, in patients with diabetes type 1 or 2, this association was not so clear[5]. Recently, artificial intelligence allowed us to find improved predictive models for CAD based on multi-variables (clinical, image, biochemical, *etc*.) such as epicardial fat quantity, measured by CT, and diabetes. Both factors are CAD risk factors[6]. However, this fat tissue also expresses or releases differential molecules in patients with diabetes[7,8]. The failure of the adipocyte's function enhances circulating glucose levels that modify and reduce proteins, lipids, or nucleic acids in a non-enzymatic reaction[9].

Advanced glycation end products and CAD

The name of these products is advanced glycation end products (AGEs) and Nε-carboxymethyl-lysine (CML), Nε-carboxyethyl-lysine, pyrraline, crossline, pentosidine, imidazolium cross-link derived from glyoxal and lysine-lysine, and imidazolium cross-link derived from methylglyoxal and lysine-lysine are some of them[10]. CML is one of the most common AGEs and can be processed from food, such as milk, bakery products, and coffee[11]. The study CORDIOPREV showed higher CML levels in those patients with established endothelial dysfunction in comparison with new T2DM [12]. But also circulating levels of AGE were associated with coronary artery calcification[13]. The preclinical atherosclerosis murine models showed that CML might increase the calcification of the plaques through muscle cell effects[14]. The AGE-RAGE signaling can activate secondary messengers (protein kinase C, mitogen-activated protein kinase, and nuclear factor kappa b)[15]. All of them are involved in proliferation or inflammation pathways. But, CML through CD36 can also enhance the macrophage-derived foam cells[16]. These findings suggested that CML can also be a mediator of CAD in patients. The results showed by Shrivastav *et al*[17] showed the association between CML and inflammatory cytokines in patients with and without diabetes. Thus, the peptides that block the RAGE pathways might be a therapeutic alternative against the proliferation and inflammation effects of CML[18]. Its quantification on patients with high risk for CAD might improve personalized medicine. The knowledge of how adiposity and non-vegetarian diet contribute to CML levels might help us to modify primary preventive strategies with consequences on CAD events.

CONCLUSION

This study contributes to the knowledge of biomarkers and therapeutic targets for diabetic patients and the identification of the phenotype with a higher risk for CAD events. This is a new avenue of personalized medicine (Figure 1).

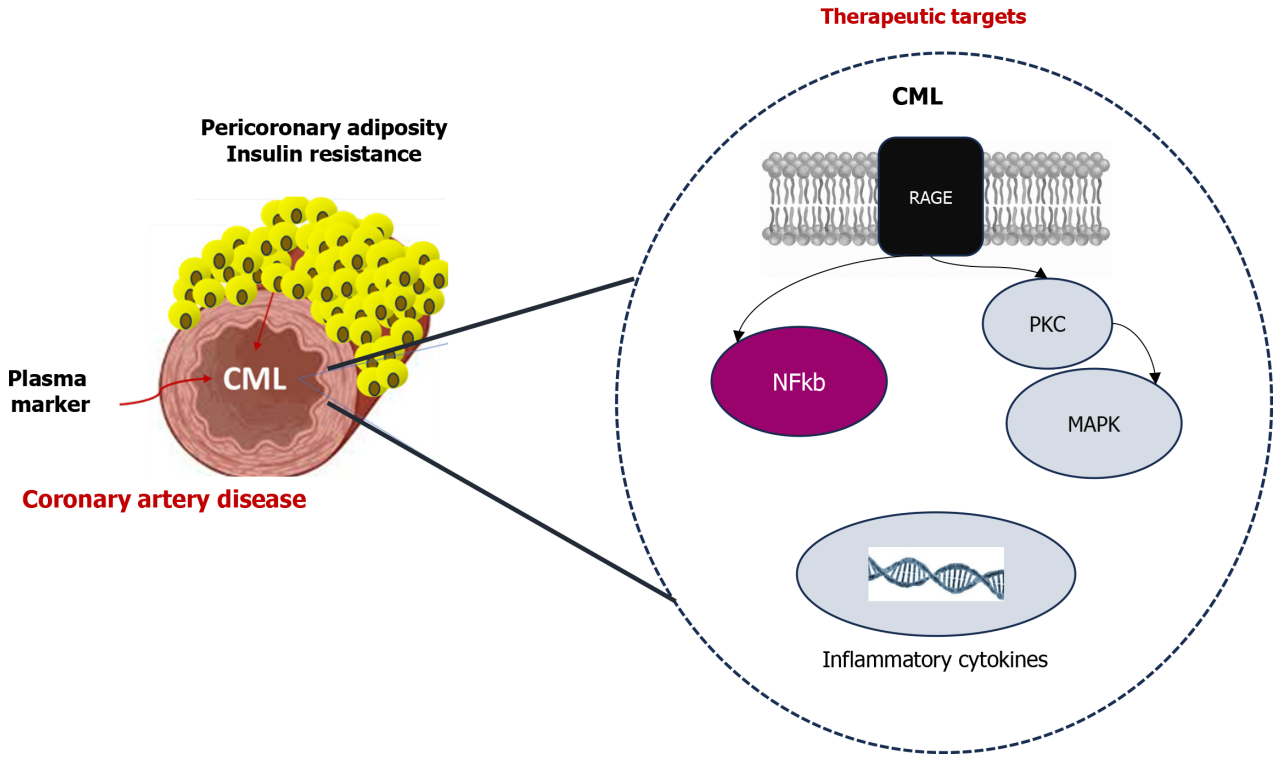

Figure 1 A summary of Nε-carboxymethyl-lysine signals transduction effects on cells. The Nε-carboxymethyl-lysine (CML) levels can be induced by an increment of adiposity, insulin resistance, and consequently, circulating glucose levels that modify molecules in a non-enzymatic way. It provokes the advanced glycation end products and CML is one of the most prevalent. But the increment of its levels might be also induced by diet. High levels of CML are markers for coronary artery disease risk. CML can also induce signal transduction and be involved in a pathological mechanism through activation of protein kinase C, mitogen-activated protein kinase, or nuclear factor kappa b, causing muscle cell proliferation or inflammatory cytokines transcription, respectively. CML can be a marker and therapeutic target. CML: Nε-carboxymethyl-lysine; PKC: Protein kinase C; MAPK: Mitogen-activated protein kinase; NFkb: Nuclear factor kappa b.

FOOTNOTES

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EDITORIAL

Non-pharmacological interventions for diabetic peripheral neuropathy: Are we winning the battle?

Dania Blaibel, Cornelius James Fernandez, Joseph M Pappachan

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Abstract

Despite the advent of relatively reliable modalities of diagnosing diabetic peripheral neuropathy (DPN), such as nerve conduction studies, there is still a knowledge gap about the pathophysiology, and thus limited available interventions for symptom control and curtailing disease progression. The pharmacologic aspect of management is mainly centred on pain control, however, there are several important aspects of DPN such as loss of vibration sense, pressure sense, and proprioception which are associated with risks to lower limb health, which pharmacotherapy does not address. Furthermore, published evidence suggests non-pharmacologic interventions such as glycaemic control through dietary modification and exercise need to be combined with other measures such as psychotherapy, to reach a desired, however modest effect. Acupuncture is emerging as an important treatment modality for several chronic medical conditions including neuropathic and other pain syndromes. In their study published in the *World Journal of Diabetes* on the potential of acupuncture to reduce DPN symptoms and enhance nerve conduction parameters, Hoerder *et al* have been able to demonstrate that acupuncture improves sensory function and that this effect is likely sustained two months after treatment cessation. Although previous studies also support these findings, larger multi-center randomized control trials including a sham-controlled arm accounting for a placebo effect are required. Overall, given the satisfactory safety profile and the positive results found in these studies, it is likely that acupuncture may become an important

aspect of the repertoire of effective DPN management.

Key Words: Diabetic peripheral neuropathy; Diabetes mellitus; Pharmacotherapy; Acupuncture; Neuropathic pain; Nonpharmacological intervention

Core Tip: Even with multiple studies examining the pathobiology and management options for diabetic peripheral neuropathy (DPN), especially the neuropathic pain, there are still large knowledge gaps in our understanding to effectively address this important clinical problem. Acupuncture is an important nonpharmacological option for several chronic medical conditions including pain syndromes. In their study published in the *World Journal of Diabetes*, Hoerder *et al* provide us the reasonable efficacy of acupuncture for the management of DPN, though we need larger multi-center randomized clinical trials for using this therapeutic intervention to enable more evidence-based clinical decision-making.

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INTRODUCTION

Peripheral neuropathy is one of the most common and difficult-to-manage complications of diabetes mellitus (DM). Distal symmetric polyneuropathy (DSPN) is the most common form of diabetic peripheral neuropathy (DPN) which affects around 50% of individuals with type 2 DM (T2DM) with disease duration > 10 years, and approximately 20% of patients with type 1 DM (T1DM) with the disease duration > 20 years[1]. Even 10%–15% of T2DM cases may have DSPN at the time of diagnosis of DM as the metabolic derangements in T2DM might have been present for several years before the actual diagnosis. Furthermore, the lack of standardized diagnostic criteria for diabetic neuropathy in the literature creates difficulty in comparing studies, even though it is imperative to initiate early management. The reason for this is the presence of peripheral neuropathy in between 25% and 62% of patients with prediabetes, which often go on to develop chronic painful DSPN[2]. In this paper, we aim to highlight current reliable methods of diagnosing and effectively managing DPN, with a focus on acupuncture as a novel non-pharmacotherapeutic option for DPN symptom alleviation.

PATHOPHYSIOLOGY

Although there is only a limited understanding of the mechanisms of development and progression of DPN based on published evidence, there are several proposed theories based on experimental data. For instance, disintegration of the myelin sheath and Schwann cells leading to axonal degeneration has been shown in nerve biopsies from animal and human models with DPN[3]. Axonal degeneration results in disruption of impulse signalling, and conduction, and creates afferent nerve axonal loss that progresses in a length-dependent fashion[3]. This is likely why long nerve fibres such as the peroneal and sural nerves are affected early in the course of DPN. Furthermore, changes in the blood-nerve barrier (BNB) function are linked to the incidence and development of DPN. Increased permeability of the BNB results in leakage of proteins such as albumin and immunoglobulin G into the endoneurium with the development of progressive oedema and subsequent ischemic nerve damage[3,4].

There is also evidence to suggest that systemic inflammation[5] and accumulation of advanced glycation end products [6] are associated with the occurrence of DPN. It is likely that hyperglycaemia leads to the upregulation of systemic inflammation and subsequent oxidative stress. Activation of various inflammatory and oxidative pathways by a chronic hyperglycaemic state in poorly controlled DM leads to the accumulation of various reactive oxygen species which may induce neuronal damage and apoptosis leading to DPN[3].

CLINICAL PRESENTATION

Although peripheral neuropathy affects 20% to 50% of patients with DM, it is still important to distinguish DPN from other disorders of the peripheral and central nervous system associated with neuropathy, medication-induced and toxic neuropathies, various vitamin deficiency states, infective conditions such as Lyme disease, and human immunodeficiency virus disease, Guillain-Barre syndrome, compressive/entrapment neuropathy, and hereditary neuropathies. In particular, the most common presentation of DPN is an insidious symmetric sensory alteration of the distal extremities that

progresses in a "stocking and glove" pattern. While numbness and diminished vibration sense and proprioception are attributed to large-myelinated fibre neuropathy, pain and decreased pinprick sensation are due to small unmyelinated fibre damage. It is important to note that most patients with DPN often exhibit large and small fibre involvement[7].

Symptoms generally begin in the toes followed by the calves and then subsequently the fingers and forearms once the symptoms ascend above knee level. Nearly a third of patients report neuropathic pain, with other common symptoms such as hyperesthesia or allodynia also being prevalent[8]. It is also important to determine whether any concurrent autonomic symptoms are present such as orthostatic hypotension, gastroparesis, or erectile dysfunction. Distinguishing DPN from central nervous system lesions is critical by verifying about symptoms and signs such as dysarthria, cranial nerve involvement, and visual disturbances. Nerve compression or radiculopathy tends to develop in an acute asymmetrical fashion, bearing in mind that emergencies such as cauda equina syndrome need to be excluded. Furthermore, patient's history may point towards a hereditary neuropathy if there is a report of childhood clumsiness or difficulty with shoe fitting[8].

While current guidelines recommend assessing DPN in patients with T2DM at diagnosis and patients with T1DM five years after diagnosis and then annually thereafter, it should be stressed that DPN is often underdiagnosed due to the lack of rapid, reliable, as well as highly sensitive and specific testing methods that can be done in the clinical setting[7].

DIAGNOSIS OF DIABETIC PERIPHERAL NEUROPATHY

Although diagnosing DPN often remains primarily clinical, several testing modalities can aid diagnosis which constitutes screening tools, quantitative sensory testing, as well as nerve conduction studies (NCS)[3]. The most widely used scoring method is the Douleur Neuropathique 4 (DN4)[9]. This technique allows the clinician to assess the signs and symptoms such as paraesthesia, hypoesthesia, as well as burning or shooting pain. It is a 10-item scoring system with a cut-off of 4 as an indication that the diagnosis is likely. DN4 has a reported sensitivity of 80% and a specificity of 91%, rendering this a reliable tool in assisting initial diagnosis[3]. There are other relatively dependable scoring metrics such as the Toronto Clinical Neuropathy Score, the Michigan Neuropathy Screening Instrument (MNSI), the Small Fiber Neuropathy and Symptoms Inventory Questionnaire, and the Neuropathy Disability scores. However, their sensitivity and specificity do not at present compare with that of the DN4[3]. Furthermore, the DN4 is a reasonably simple scoring tool, likely contributing to its popular use amongst clinicians.

Quantitative sensory testing is often a useful aid to sign and symptom evaluation in establishing DPN diagnosis. The evidence does suggest that combining a scoring metric in history taking, along with quantitative sensory testing such as with a tuning fork and the 10 g monofilament, reflects greater sensitivity and specificity in overall means of accurate diagnosis.

The gold standard for diagnosis of DPN however, remains NCS[3]. The test can assess myelinated α and β large fibres through velocity of nerve conduction, amplitudes, and latencies. Since DPN affects the long fibres of the lower extremities, it is the plantar, peroneal, and sural nerves that are often evaluated. The gold standard for identifying small nerve abnormalities is the intraepidermal nerve fibre density, whereby immunohistochemical testing is conducted on a distal skin biopsy and stained small nerve fibres are counted and compared to standardized values[4]. It should be noted that a systematic evaluation of DPN comprising detailed history taking, using a quantifiable scoring metric, sensory testing through a pinprick, tuning fork, and monofilament, along with NCS is more likely to yield a more accurate diagnosis.

PHARMACOTHERAPY AND ITS LIMITATIONS

Pharmacotherapy is limited in its capacity to effectively treat DPN as there is a lack of disease-modifying agents, with the sole aim of pharmacotherapy at present being pain control. Furthermore, multiple clinical trials have indicated that although achieving glycaemic control has shown some beneficial effects in ameliorating DPN in T1DM, this has not been the case for patients with T2DM[7]. There are currently four classes of drugs approved for the treatment of DPN-related pain including tricyclic antidepressants, serotonin-norepinephrine reuptake inhibitors, gabapentanoids, and sodium channel blockers. Unfortunately, given these medications have similar effect sizes and differences within a class are likely minimal to non-existent[7], clinicians usually prescribe according to subjective patient experience on questionnaires, while attempting to implement cost-effective and tolerable therapies.

Moreover, clinicians often have to prescribe multiple medications in order to achieve relative pain control which increases the pill burden on patients who often experience medication side effects. Recently there has been research investigating the use of sodium glucose cotransporter 2 inhibitors to treat DPN, with it showing promising effectiveness in T1DM, however, this has only been demonstrated in animal models[7,10]. With regards to topical therapy, capsaicin is the most widely studied and interestingly carries a similar effect size as oral medication. Nevertheless, given the effect size of oral and topical therapy is limited, with approximately only 1 in 7 patients with DPN reporting effective pain relief [7], treating patients should focus on starting a combination of oral and topical therapy while titrating dosages as the pain worsens with disease progression.

NON-PHARMACOTHERAPEUTIC OPTIONS

The clinical management of DPN has often focused on weight loss through dietary modification and exercise, orthotic footwear, and annual foot examinations, as well as patient education and psychological intervention. For instance, in the Look Ahead study, 5145 diabetic patients were randomized to an arm that focused primarily on weight loss as an intervention[11]. This study showed that the dietary weight loss did reflect symptom improvement on the MNSI questionnaire. Another recent observational study also showed that weight loss seems to show symptom reduction on the MNSI questionnaire[12]. Therefore, it is likely that weight loss does show a potential in managing patients symptomatically, however, weight loss needs to be combined with other interventions to achieve a disease-modifying effect. With regards to behavioural intervention, it has been demonstrated that cognitive behavioural therapy, when combined with exercise has shown potential for effective symptom management, with similar evidence reported in studies on chronic illnesses such as fibromyalgia[13].

Neuro-modulatory treatment modalities have been an important nonpharmacological therapeutic intervention for various pain syndromes in the past few decades. The basic principle of this treatment is altering the electrical signals in the pain-subserving neural pathways to increase the pain perception threshold or by stimulation of neural transmitters with pain inhibition potential. Transcutaneous electrical nerve stimulation was found to be effective in managing patients with painful DPN in the past[14]. A recent systematic review reported good efficacy of spinal cord electrical stimulation (SCS) in improving the symptoms of painful neuropathies[15]. Apart from various techniques used in SCS, dorsal root ganglion stimulation is another modality of pain control in painful DPN[16].

ACUPUNCTURE TREATMENT FOR CHRONIC CONDITIONS

Acupuncture is one of the non-pharmacotherapeutic options for the treatment of several chronic painful states including DPN. Historically, acupuncture has been used to treat various conditions such as chronic migraine, carpal tunnel syndrome, fibromyalgia, as well as other musculoskeletal pain-related conditions. The interest in acupuncture is that it is a non-invasive and cost-effective therapy, with a reasonable safety profile. In a meta-analysis of data involving 20827 patients from 39 trials, it was shown that acupuncture was superior to both the sham and no acupuncture controls for each pain condition[17]. Furthermore, the authors demonstrated that although there is a minor decrease of 15% in treatment effect after one year, the efficacy of acupuncture is maintained over time. In particular, there have been studies on the effectiveness of acupuncture on chemotherapy-induced neuropathy in cancer patients. Therefore, it is evident that acupuncture can be used as a safe means to alleviate symptoms of peripheral neuropathy across various patient populations.

ACUPUNCTURE TREATMENT FOR DPN

Acupuncture has been postulated as a safe and effective means for managing DPN. In a recent study of various systematic reviews, it has been illustrated that acupuncture improves nerve conduction and clinical symptoms[18]. These results have been echoed by another meta-analysis which has shown that most randomized controlled trials favour the use of acupuncture over the non-acupuncture control for minimizing neuropathic pain[19]. ACUDIN trial was a recent three-armed randomized placebo-controlled trial, among patients with confirmed DPN evaluated over a series of 10 consecutive weeks. The trial was able to demonstrate that acupuncture treatment improved the amplitude of the sural nerve action potential by 1.95 while only 0.5 was noticed in the placebo group[20]. Furthermore, the sural nerve conduction velocities improved significantly by a mean of 13.5 m/s in the acupuncture group compared to placebo lase with 3.4 m/s. This suggests that acupuncture has the potential to not only improve patient-reported outcomes on questionaries or examination scores but also nerve conduction parameters.

In their randomized control trial published in the *World Journal of Diabetes*, Hoerder *et al*[21] focused on the use of acupuncture to manage hypesthesia, numbness, and loss of sensory function in patients with DPN. This is an especially important area for investigation as hypesthesia, numbness, and loss of sensory function are implicated in falls, foot injury, and ulceration, as well as lower limb amputation with disease progression. Immobility and reduced independence from DPN would likely have a negative impact on overall patient morbidity and mortality. Hoerder *et al*[21] have been able to demonstrate that after a series of acupuncture sessions in those with moderate to severe DPN symptoms, patients showed improved sensory function, as well as reduced dysesthesia on symptom inventory questionnaires and neurological examination scores. This was especially evident between week 8 and week 16 of treatment, whereby patients reported a reduction in numbness of about 32%[21]. Impressively the acupuncture effects seem to have lasted nearly 2 months post-treatment compared to the control group. However, a placebo effect has not been considered in this regard. This study does have a few limitations, such as nerve conduction parameters not being shown with the use of the DPN-check, the lack of blind clinical assessors, as well as a relatively small sample size. Future studies should focus on a large sample size in a double-blind randomized clinical trial (RCT), whereby NCS are employed to provide more accurate information on any change in nerve conduction following acupuncture therapy. Figure 1 shows currently available management options for DPN.

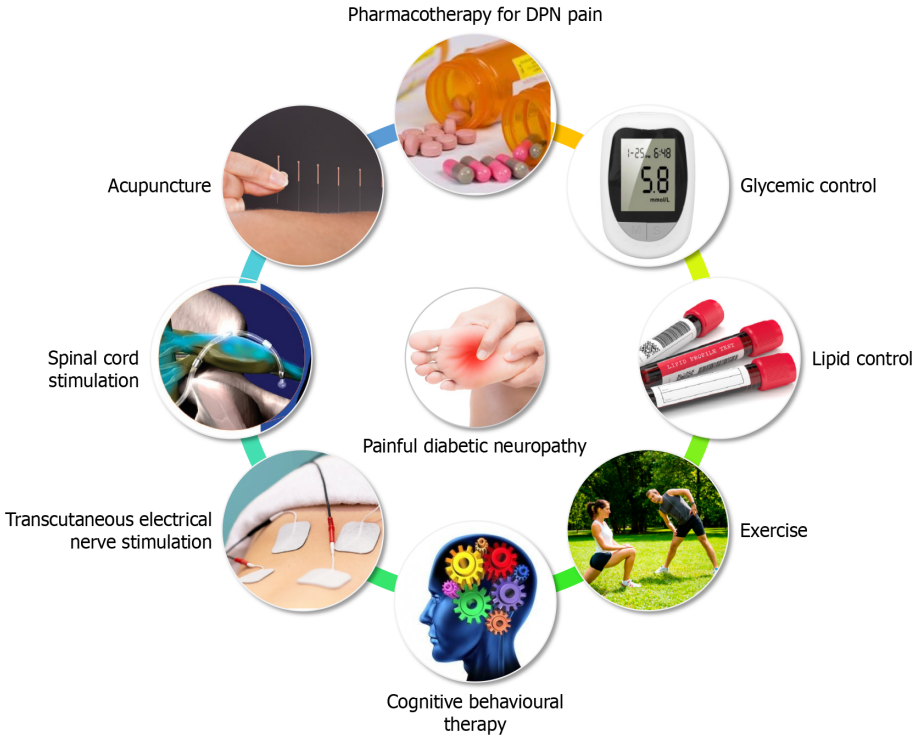

Figure 1 The therapeutic options for the management of painful diabetic neuropathy. DPN: Diabetic peripheral neuropathy.

EMERGING RESEARCH AND POTENTIAL NOVEL THERAPEUTIC OPTIONS

Historically, research on the pathogenesis of DPN has focused on the role of hyperglycaemia and hyperlipidaemia in disrupting mitochondrial function through the accumulation of reactive oxygen species, ultimately resulting in neuronal apoptosis and axonal failure[22]. However, understanding the microenvironment of the neuron during DPN development and the role of various cellular components such as Schwann cells and macrophages is critical for testing and innovating targeted therapies. For instance, novel research is now capable of deriving Schwann cells from human pluripotent stem cells that mimic the molecular features of primary Schwann cells and are capable of myelination *in vivo* and in vitro. Interestingly, researchers were able to demonstrate that bupropion, an antidepressant, counteracts glucotoxicity, as well as prevents sensory dysfunction and Schwann cell apoptosis in mice[23]. This method is an excellent modality for screening the effectiveness of potential drug candidates as well as studying whether certain pharmacotherapies inhibit DPN development. In addition, this will likely also lead to an enhanced understanding of the primary biochemical pathways involved in disease onset and progression.

There have also been several studies in the literature on non-pharmacological interventions to improve sensory function and reduce patient pain scores, specifically focusing on diet and physical activity. For instance, some recent studies have reported that a keto diet along with exercise has the potential to prevent and reverse the effects of DPN[24], while others have shown that switching from a diet rich in saturated fats to a diet rich in plant-based unsaturated fats and fish oil restores nerve function and counters axonal mitochondrial dysfunction[22]. Future studies must analyse various permutations of both pharmacological and non-pharmacological interventions in DPN patients. This will assist in determining the most effective holistic first-line treatments for patients, and likely therefore significantly enhance patient morbidity and mortality outcomes.

CONCLUSION

DPN is one of the most common chronic complications of diabetes affecting up to a half of the patients in their diabetes journey. It is imperative that DPN is diagnosed and managed early, in order to preserve patients' foot health and thus the quality of life. It would be interesting if future RCTs and meta-analyses investigated which combinations of quantitative testing allow for the earliest accurate diagnosis of DPN, as well as which combination of pharmacologic as well as non-pharmacologic intervention seems to be the most effective at managing symptoms and reducing disease progression. More effective pharmacologic and nonpharmacological treatments are to be developed to improve the care of patients with this crippling chronic ailment.

Future studies should also focus on randomized and sham-controlled clinical trials in order to assess the effectiveness of acupuncture on several outcomes such as neurological testing and NCS. Other endpoints should include subjective patient experience, as well as neurological examination scores. It is evident that acupuncture seems to be a safe and effective modality of improving patient symptoms as well as nerve conduction, warranting its inclusion as a potential

recommendation for the therapy of patients diagnosed with DPN. Future research targeting molecular-level disease-modifying therapy based on the pathogenic mechanisms of DPN is expected to improve our therapeutic strategies against this enigmatic disease.

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EDITORIAL

Effect of bariatric surgery on metabolism in diabetes and obesity comorbidity: Insight from recent research

Hui-Hong Tang, Dong Wang, Cheng-Chun Tang

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Abstract

Obesity is a prevalent cause of diabetes mellitus (DM) and is a serious danger to human health. Type 2 DM (T2DM) mostly occurs along with obesity. Foodborne obesity-induced DM is caused by an excessive long-term diet and surplus energy. Bariatric surgery can improve the symptoms of T2DM in some obese patients. But different types of bariatric surgery may have different effects. There are some models built by researchers to discuss the surgical procedures' effects on metabolism in diabetes animal models and diabetes patients. It is high time to conclude all this effects and recommend procedures that can better improve metabolism.

Key Words: Bariatric surgery; Obesity; Diabetes; Animal models; Diabetes patients

Core Tip: Bariatric surgery is a type of treatment that can improve the metabolic status and prognosis of patients with obesity and diabetes comorbidities. Bariatric surgery could alleviate obesity and has a positive effect on metabolism in diabetes animal models and diabetes patients, suggesting that the recommended frequency of bariatric surgery for diabetic and obese comorbid patients should be increased.

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INTRODUCTION

Obesity and type 2 diabetes mellitus (T2DM) typically co-occur. The pathophysiology of obesity is primarily caused by insulin resistance, hyperinsulinemia, hormonal dysregulation, and systemic inflammation[1]. Bariatric procedures are an option for those who want to help themselves reduce weight. Sleeve gastrectomy (SG), gastric banding, and Roux-en-Y gastric bypass (RYGB) are common bariatric operations performed in clinical practice. Studies have been conducted on how bariatric surgery affects diabetes metabolism. Recently, a journal article published in this journal titled, "Impact of bariatric surgery on glucose and lipid metabolism and liver and kidney function in food-induced obese diabetic rats," conducted basic research on this topic.

Studies on the effect of bariatric surgery on metabolism in diabetes

Several clinical trials have repeatedly demonstrated the critical role that surgery plays in improving glucose homeostasis and initiating remission. Many large cohort studies comparing the two approaches to obesity management indicate that patients undergoing bariatric surgery have a higher chance of achieving remission of diabetes than those who only receive standard obesity treatment[2-6]. Patients with diabetes may undergo a brief course of therapy after bariatric surgery. The factors most frequently associated with remission are younger age, a higher C-peptide level, diabetes for less than 4 years before the surgery, and relying only on diet or oral medication to treat the illness[7-10]. In a joint statement, the American Diabetes Association, Diabetes United Kingdom, the Chinese Diabetes Society, Diabetes India, and the International Diabetes Federation urged patients with class I obesity (body mass index: 30.0-34.9 kg/m^2) and poorly controlled hyperglycemia despite receiving the best possible medical care, including insulin, to consider bariatric surgery [11]. A very small proportion of patients may continue to experience a protracted remission. Research assessing long-term results has shown that individuals who achieve diabetic remission have a recurrence incidence of more than 50%[6,12, 13]. From the above studies, it can be seen that bariatric surgery markedly improves blood sugar control in patients with diabetes, although the improvement is not significant for long-term diabetes management.

Similar to employing calorie restriction to achieve weight loss, bariatric surgery results in an improvement in insulin sensitivity, which is a crucial component of the pathogenesis of diabetes[14-16]. One feature that appears before weight loss after bariatric surgery is the rapid improvement in glucose management. Many patients are discharged insulin-free, even though they had needed hundreds of units of insulin before surgery[17]. The above studies have shown that bariatric surgery can improve the patient's insulin sensitivity, thereby allowing the patient to use a reduced amount of insulin after surgery.

Changes in the repertoire of systemic bile acids and elevated glucagon-like peptide 1, a circulating incretin hormone, have been reported following the surgery[18]. Bariatric surgery preserves β cell function and coordinates islet activity, which partially improves glycemic control. Changes in circulating glucagon-like peptide 1 levels can indirectly affect β cells through changes in body weight, or they can act directly[19]. Bile acids are metabolites generated from cholesterol that act as detergents to facilitate the absorption of vitamins and lipids and act as ligands for host receptors[20]. The etiology of T2DM is linked to chronic inflammation associated with obesity[21]. Furthermore, pancreatic fatty acid production following RYGB surgery is essential for β cell function during calorie restriction[22]. The changes in lipid metabolism and the reduction of inflammation caused by bariatric surgery also have an important impact on remission in patients with diabetes.

Bariatric surgery reverses endothelial dysfunction by improving nitric oxide availability and inhibiting vascular oxidative stress; it also serves as an effective anti-inflammatory strategy by mitigating interferon-γ-mediated adipose tissue inflammation[23]. Changes in the jejunal Roux limb mRNA and lncRNA expression patterns initiate neuromodulation and endocrine-related pathways *via* the gut-brain axis that is essential for remission of T2DM following metabolic and bariatric surgery[24]. In addition, a blood signature of diabetes reversal in mice highlights new miRNA–gene interactions in the pancreatic islets during the resolution of diabetes following bariatric surgery[25,26]. Therefore, it can be seen that oxidative stress, neuromodulation, and endocrine regulation also affect remission in patients with diabetes after bariatric surgery.

Vertical SG surgery in the UC Davis T2DM rat model postponed the onset of diabetes, which is partially independent of a decrease in body weight[27]. Experimental metabolic surgery significantly lowers albuminuria in a rat model of diabetic kidney disease[28,29]. Reductions in podocyte stress, glomerulomegaly, and glomerulosclerosis post-RYGB in Zucker diabetic fatty rats indicate improved glomerular histology. Quantifiable decreases in podocyte foot process effacement indicate an improvement in glomerular ultrastructure post-RYGB and post-SG. Interestingly, a more noticeable decrease in proteinuria is observed when RYGB is used instead of SG. In addition, research on humans suggests that RYGB may better regulate metabolism than SG[30]. RNA sequencing has been used to characterize the transcriptional program underlying these structural changes at the pathway level. This program has been linked to a considerable decrease in the activation of fibrotic and inflammatory responses. In Zucker diabetic fatty rats, weight loss and improvements in glycemia after RYGB surgery are accompanied by normalization of glomerular tuft size, decreases in desmin expression by podocytes, and preservation of the morphology of the podocyte foot process compared to sham-operated control animals[31]. It can be seen that bariatric surgery also improves the renal function in the diabetic obese rat model, which can improve the glomerular structure.

Highlights of the chosen article

This study was selected to provide commentary because it has noteworthy findings with clinical implications. Diabetes mellitus (DM) typically develops in response to obesity and poses a major threat to human health. T2DM often coexists with obesity. Excessive long-term eating and excess energy are the causes of foodborne obesity-induced DM. Some obese

people may find that their T2DM symptoms are alleviated after bariatric surgery; however, the outcomes of various bariatric procedures vary. In this study, the effects of various bariatric surgeries on the prognosis of patients with diabetes and obesity were explored.

This study showed that bariatric surgery affects liver and kidney function, as well as glucose and lipid metabolism, by modulating the PKCβ/P66shc pathway in food-derived obese diabetic rats. The PKCβ/P66shc pathway plays a role in intracellular crosstalk and signal transduction[32] and has received considerable attention because of the connection between excess nutrient intake and obesity[33]. Bariatric surgery to alleviate obesity affects metabolism and may provide a new way of solving diabetes and obesity comorbidities and offer a novel treatment for foodborne obesity-induced diabetes. The PKCβ/P66shc pathway explored herein is an extensively studied oxidative stress pathway, suggesting that alleviating oxidative stress may be a possible way to ameliorate diabetes and obesity comorbidities.

This study also analyzed the pros and cons of various bariatric surgeries, which is essential in clinical use when surgeons are choosing surgical modalities. RYGB tends to result in a tiny wound with low risk, favorable prognosis, lower recurrence rate by reducing islet cell apoptosis, an increase in insulin secretion, and restoration of islet function. However, RYGB might lead to excessive blood sugar, anastomosis inflammation locally, and stomach discomfort in mice. Also, it might result in intestinal adhesion, infection, poor closure of the surgical incision, gastric paresis, gastrointestinal dysfunction, abdominal distension, and incapacity to eat. SG can effectively control T2DM, obesity, and the risk of obesity-related cardiovascular and cerebrovascular complications by reducing the volume of the stomach, reducing weight, and improving T2DM. However, SG completely removes the fundus of the stomach and may increase the risk of developing gastroesophageal reflux disease. Gastric banding also reduces weight by reducing food intake. The surgical damage is minimal, and the postoperative recovery is fast. However, the surgical effect is suboptimal, resulting in limited weight loss. The above results suggest that in clinical settings, the selection of the type of bariatric surgery according to the patient's individual situation will result in different postoperative complications and personal perceptions, and may also improve patient surgical satisfaction.

CONCLUSION

Diabetes and obesity are increasingly threatening human health. The traditional five-step approach to diabetes, comprising patient education, dietary control, medication, exercise therapy, and self-monitoring management, is not universally effective due to physiological, behavioral, and economic barriers. Bariatric surgery is increasingly recognized as an effective treatment for patients with T2DM and obesity. While surgery does not solve the underlying problem of oversupply of energy and does not cure the disease, it significantly reduces the burden on patients. Elucidating the mechanisms of metabolic function in patients will improve healthcare professionals' understanding of the disease. Bariatric surgery represents both an enlightening scientific model and an effective treatment to address the diabetes crisis. In conclusion, bariatric surgery alleviates obesity and has a positive effect on metabolism in diabetes animal models and patients with diabetes, suggesting that the recommended frequency of bariatric surgery for patients with diabetes and obesity should be increased.

FOOTNOTES

Co-corresponding authors: Dong Wang and Cheng-Chun Tang.

Author contributions: Tang HH, Wang D, and Tang CC conceived, designed, and refined the study; Tang HH drafted the manuscript; Wang D and Tang CC contributed equally to this work as co-corresponding authors. The reasons for designating Wang D and Tang CC as co-corresponding authors are as follows. First, they both participated in choosing the idea of the study. Second, they both revised the manuscript. Third, they both are responsible for the study. In summary, we believe that designating Wang D and Tang CC as co-corresponding authors of is fitting for our manuscript as it accurately reflects our team's collaborative spirit, equal contributions, and diversity.

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EDITORIAL

Application and management of continuous glucose monitoring in diabetic kidney disease

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Abstract

Diabetic kidney disease (DKD) is a common complication of diabetes mellitus that contributes to the risk of end-stage kidney disease (ESKD). Wide glycemic variations, such as hypoglycemia and hyperglycemia, are broadly found in diabetic patients with DKD and especially ESKD, as a result of impaired renal metabolism. It is essential to monitor glycemia for effective management of DKD. Hemoglobin A1c (HbA1c) has long been considered as the gold standard for monitoring glycemia for > 3 months. However, assessment of HbA1c has some bias as it is susceptible to factors such as anemia and liver or kidney dysfunction. Continuous glucose monitoring (CGM) has provided new insights on glycemic assessment and management. CGM directly measures glucose level in interstitial fluid, reports real-time or retrospective glucose concentration, and provides multiple glycemic metrics. It avoids the pitfalls of HbA1c in some contexts, and may serve as a precise alternative to estimation of mean glucose and glycemic variability. Emerging studies have demonstrated the merits of CGM for precise monitoring, which allows fine-tuning of glycemic management in diabetic patients. Therefore, CGM technology has the potential for better glycemic monitoring in DKD patients. More research is needed to explore its application and management in different stages of DKD, including hemodialysis, peritoneal dialysis and kidney transplantation.

Key Words: Diabetic kidney disease; Continuous glucose monitoring; Glycemic monitoring; Hemodialysis; Peritoneal dialysis; Kidney transplantation

Core Tip: Continuous glucose monitoring (CGM) shows the strength of providing a glycemic profile in diabetic kidney disease (DKD). This article summarizes the use of CGM in early and advanced stages of DKD, including hemodialysis, peritoneal dialysis, and kidney transplantation. CGM may be considered an alternative or complement to measurement of hemoglobin A1c in some contexts.

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INTRODUCTION

Diabetes mellitus (DM) is a chronic disorder characterized by sustained hyperglycemia and its prevalence has caused an increased healthcare burden worldwide[1]. Long-term hyperglycemia and metabolic alterations can lead to various diabetic complications, causing damage to tissues and organs[2]. Diabetic kidney disease (DKD) is one of the major microvascular complications, accounting for 20%-50% among diabetic patients[3]. DKD has been considered as a primary category of chronic kidney disease (CKD) and a leading cause of end-stage kidney disease (ESKD), contributing to the large physical and financial burden globally[4]. DKD is classified in accordance with progressively increased albuminuria (≥ 30 mg/g) and decline in estimated glomerular filtration rate (< 60 mL/min/1.73 m^2)[5].

The risk of hypoglycemia and hyperglycemia is predominantly increased in patients with DKD and particularly at advanced stages. Various factors contribute to glycemic variation in DKD, including impaired renal gluconeogenesis, defective renal clearance of insulin, elevated insulin resistance, and diminished β-cell function[6]. With progressive decline of renal function, initiation of peritoneal dialysis or hemodialysis could markedly affect glycemic variability because the glucose content of dialysates can alter daily glucose profiles[7,8]. The conventional glycemic marker glycated hemoglobin A1c (HbA1c) is limited for the prediction of daily glycemic variability and acute hyperglycemia/hypoglycemia. Moreover, its accuracy and precision are weakened with advanced CKD, and particularly among patients with dialysis. Alternative glycemic indicators, such as glycated albumin or fructosamine, have not been fully validated and applied because the cost and difficulties of implementation in daily practice. Therefore, optimal glycemic control is faced with challenges in patients with DKD.

Continuous glucose monitoring (CGM) is one of the innovative technologies for glycemic monitoring in the past 100 years[9]. CGM devices provide multiple data, including proportion of time-in-target range (TIR), glucose variability and glucose management indicator (GMI), which enable patients to respond immediately prior to acute glycemic events and assist clinicians to adjust appropriate treatment for patients[10]. Two main types of CGM system technologies, real-time CGM (rtCGM) and intermittently scanned CGM (isCGM), are currently available for use. rtCGM systems automatically transmit the data to a receiver, while isCGM systems require the patient to swipe the receiver to access current and historical glycemic files[11]. Emerging studies on the use of CGM suggest its potential for more precise glucose monitoring in patients with DKD compared with other glycemic markers[6,12,13]. The latest Kidney Disease Improving Global Outcomes (KDIGO) guideline advocates that use of CGM may help prevent hypoglycemia and improve glycemic control for patients in whom HbA1c is not compatible with directly measured glycemic levels or clinical symptoms[14].

APPLICATION OF CGM IN NONDIALYSIS PATIENTS WITH DKD

Various studies have assessed correlation between CGM metrics and conventional glycemic markers including HbA1c, glycated albumin and fructosamine during different DKD stages. The beneficial effect of CGM on glycemic control in patients with early stages of CKD is comparable to that in the general population with diabetes. With decreased renal function, the accuracy of HbA1c tends to fall in advanced DKD, partly due to anemia and treatment with iron supplements or erythropoietin-stimulating agents[15,16].

A recent study by Lu *et al*[17] has assessed the association between HbA1c and CGM metrics among patients with different stages of DKD. The correlation between HbA1c and GMI was attenuated with impaired renal function as shown in patients with CKD KDIGO 1-2 stages (r = 0.576) and stage 3 (r = 0.266). HbA1c was not significantly correlated with GMI in CKD KDIGO 4-5 stages (r = 0.296, P = 0.079). Ling *et al*[18] also evaluated the relationship between HbA1c and CGM metrics in moderate-to-advanced DKD (CKD KDIGO 3b to 5), which found correlations between GMI and HbA1c attenuated with advancing DKD [CKD KDIGO 3b (r = 0.68), CKD KDIGO 4 (r = 0.52), CKD KDIGO 5 (r = 0.22)]. HbA1c did not correlate with duration of hypoglycemia in any DKD stage, although it may have been associated with TIR and time in hyperglycemia in DKD (CKD KDIGO 3b-4). Likewise, Lo *et al*[19] indicated that HbA1c correlated well with mean CGM glucose in CKD KDIGO 3 (r = 0.79) but gradually weakened in CKD KDIGO stage 4-5 (r = 0.34). Vos *et al*[20] also observed poor correlation between HbA1c and CGM in DKD (CKD KDIGO stage 4-5, r = 0.38), while they found glycated

albumin correlated significantly with CGM mean glucose in patients with DKD (CKD KDIGO stage 4-5, r = 0.54). Therefore, their study suggested that glycated albumin is more accurate for assessment of glycemia compared with fructosamine and HbA1c in advanced DKD. However, a recent prospective cohort study conducted by Zelnick *et al*[12] suggested that HbA1c is no more variable and less biased than glycated albumin and fructosamine in patients with DKD (CKD KDIGO stage 3-5). They observed similar correlations of these glycemic biomarkers with CGM mean blood glucose among patients with DKD (CKD KDIGO stage 3-5) (HbA1c, r = 0.78; glycated albumin, r = 0.78; fructosamine, r = 0.71), but none of them captured any incidence of acute glycemic variability as indicated by CGM devices. Oriot *et al*[21] investigated the discordance between HbA1c and CGM-derived metrics in DKD individuals, which showed higher HbA1c levels in this population and suggested that GMI data are more precise for monitoring glycemia. Similarly, Yoshii *et al*[22] reported that higher HbA1c levels did not always protect against hypoglycemic episodes as performed by CGM devices. CGM-measured hypoglycemia is frequent in patients with DKD or CKD without diabetes[23,24]. Ushiogi *et al*[24] observed that only two hypoglycemia symptoms were reported among 366 patients during CGM measurements, but hypoglycemia occurred in 41% of DKD participants and 48% of CKD patients without diabetes according to CGM detection. Similarly, a retrospective cohort study performed in 823 diabetic patients indicated that hypoglycemic events were negatively correlated with renal function, suggesting the role of TIR, especially nocturnal TIR, in the evaluation of DKD progression[25]. Apart from deficient kidney gluconeogenesis and counter regulatory hormone responses, hypoglycemic incidence is associated with impaired clearance of antidiabetic agents such as insulin and/or sulfonylureas [26]. Therefore, using CGM-derived metrics to complement HbA1c analysis is beneficial for patients with DKD and treated with insulin and/or sulfonylureas to avoid hypoglycemic episodes regardless of HbA1c levels. The Pearson correlation between CGM metrics and glycemic biomarkers on different stages of CKD in diabetic patients is summarized in Table 1.

APPLICATION OF CGM IN DIABETIC PATIENTS ON DAILYSIS

Diabetic patients with ESKD have a wide glycemic variability during dialysis. Patients treated by hemodialysis have an increased risk of hypoglycemia, while patients with peritoneal dialysis more frequently have hyperglycemia[27]. The KDIGO 2022 guideline highlights that the precision of HbA1c falls with advanced CKD, particularly among patients treated by dialysis[14]. However, currently there is no consensus on CGM use in diabetic patients treated by dialysis.

Emerging studies have investigated CGM in the context of DKD with hemodialysis. CGM may improve glucose control and optimize therapeutic adjustments without increased risk of hypoglycemia[28]. The mean absolute relative difference (MARD) is commonly used to assess the accuracy of CGM sensors and MARD with good accuracy should be < 10% as recommended[29]. A recent study by Avari *et al*[30] compared the real-time and isCGM in 40 patients undergoing hemodialysis, and suggested that isCGM (MARD 11.3%) was more reliable than rtCGM (MARD 22.7%) for glucose monitoring. Hissa *et al*[31] showed that interstitial glucose detection by CGM devices was in good concordance with capillary measurements at the beginning of the dialysis session (MARD 16.5%-19%). The correlation, however, was weakened in later sessions (MARD 25.3%-28.8%), probably due to increased inflammatory response to sensor insertion, loss of dialysis fluid, weight changes between dialysis sessions, and anemia. In line with the study of Hissa *et al*[31], another study of 41 participants undergoing hemodialysis demonstrated that the accuracy of CGM sensor glucose levels deteriorated with duration of use from the first week (MARD 13.8%-21%) to the second week (MARD 24.5%-36.1%)[32]. They observed that MARD correlated negatively with dry weight, body mass index, hemoglobin and hematocrit after hemodialysis, which may have affected the differences between CGM and capillary glucose levels. In addition, Villard *et al*[33] compared the accuracy of CGM with capillary and venous blood glucose measurements in 20 diabetic patients on hemodialysis (MARD 13.8% and 14.4%, respectively), suggesting overall performance of CGM appears reasonably accurate and relevant for clinical use. However, MARD does not provide information about dynamic changes in glycemia or hypo-/hyperglycemia incidence; therefore, it should not be used as the sole parameter to evaluate CGM systems[34]. The study by Hayashi *et al*[7] found great glycemic variability during hemodialysis and > 20% of the participants experienced asymptomatic hypoglycemia, which is more frequent than currently recognized. Compared with HbA1c and glycated albumin, CGM values favors physicians for awareness of hemodialysis-related hypoglycemia. Bomholt *et al*[35] also investigated glycemic variations on and off dialysis in patients with T2DM using CGM. They observed that patients developed intradialytic hypoglycemia despite the use of glucose-containing dialysate, indicating that hypoglycemia is a risk during hemodialysis. The hemodialysis-related hypoglycemia may be associated with increased erythrocyte uptake of glucose and improved insulin sensitivity due to alleviation of uremia and correction of acidosis[36,37]. The use of CGM contributes to avoiding hypoglycemic events in hemodialysis, which are frequently asymptomatic and potentially severe. However, the accuracy of CGM should be improved with the progress of hemodialysis and further studies are needed to avoid influencing factors as mentioned above.

Glucose-based dialysate containing 100-300 g glucose is widely used in peritoneal dialysis. Therefore, patients undergoing peritoneal dialysis are prone to hyperglycemia due to glucose absorption from dialysate *via* the peritoneal cavity[38]. Additionally, with increasing use of glucose substitute dialysate and insulin treatment, peritoneal dialysis patients are also at risk of developing hypoglycemia. CGM may contribute to detection of asymptomatic glucose variations by hypertonic exchanges in this population. A recent study by Ng *et al*[8] showed rtCGM detection rates for hyperglycemic and hypoglycemic events were 96.5% and 60%, respectively. They suggested the accuracy and reliability of rtCGM across a wide range of glucose levels in peritoneal dialysis patients (MARD 10.4%). Notably, its accuracy was not affected by acidosis, urea levels and volume overload. Likewise, Ling *et al*[39] demonstrated that rtCGM detection was accurate in peritoneal dialysis with an overall MARD of 10.4%, and almost not influenced by overloaded volume,

Table 1 The Pearson correlation between continuous glucose monitoring metrics and glycemic biomarkers on chronic kidney disease (number of participants)

CKD stages	1-2	3a	3b	4	5	Ref.
HbA1c	0.576 (64)	0.266 (56)	0.266 (56)	0.296 (36)	0.296 (36)	Lu *et al*[17]
			0.68 (33)	0.52 (43)	0.22 (14)	Ling *et al*[18]
		0.79 (14)	0.79 (14)	0.34 (29)	0.34 (29)	Lo *et al*[19]
				0.38 (25)	0.38 (25)	Vos *et al*[20]
		0.78 (80)	0.78 (80)	0.78 (80)	0.78 (80)	Zelnick *et al*[12]
Glycated albumin				0.54 (25)	0.54 (25)	Vos *et al*[20]
Fructosamine		0.78 (80)	0.78 (80)	0.78 (80)	0.78 (80)	Zelnick *et al*[12]
		0.71 (80)	0.71 (80)	0.71 (80)	0.71 (80)	Zelnick *et al*[12]

CKD: Chronic kidney disease; HbA1c: Hemoglobin A1c.

body composition and anemia. Similar to those in hemodialysis, HbA1c is incapable of indicating acute glycemic variations that frequently occur in this population. Qayyum *et al*[40] observed that a high incidence of asymptomatic hypoglycemia was detected by CGM, but not reflected by HbA1c. They found three of 15 patients with HbA1c > 9% still had significant hypoglycemia. Additionally, the study of Bomholt *et al*[41] showed that mean glucose level was underestimated by HbA1c when compared with CGM, indicating the strength of CGM in glycemic control in peritoneal dialysis patients. A number of factors such as glucose concentration of dialysate, dwell time, and peritoneal membrane transport status may affect glycemic pattern during peritoneal dialysis[42]. Research on CGM in the peritoneal dialysis population may provide comprehensive glycemic profiles and facilitate individualized therapeutic adjustment.

APPLICATION OF CGM IN KIDNEY ALLOGRAFT RECIPENTS

Perioperative and post-transplant hyperglycemia is common and severe in kidney allograft recipients because > 20% of them have ESKD caused by long-term diabetes. Strict glycemic control is crucial for patients to prevent *de novo* post-transplant diabetes or complications of previous diabetes in the transplanted kidney and direct glucose monitoring is more beneficial for providing information on glycemic variability and warning of acute incidents[43]. Jo *et al*[44] investigated CGM applied by participants 2 wk before and 2 wk after kidney transplantations. The CGM system provided an overall hyperglycemic profile, which showed a hyperglycemic tendency, higher mean glucose levels and increased GMI from before to after intervention. A randomized study of 40 patients assessed the use of CGM devices during the first 5 d after kidney transplantation, suggesting that CGM significantly reduced the incidence of hyperglycemic episodes and median glucose levels without increasing hypoglycemic events[45]. Similarly, Jin *et al*[46] investigated glucose profiles and the degree of hyperglycemia after kidney transplantation for 1 month. They observed hyperglycemia over fasting or postprandial glucose standard occurred in 42.1% during the early period after operation, except for patients with pre-existing diabetes. However, more studies involving CGM performance at regular intervals based on different perioperative and post-transplant times are needed.

CONCLUSION

The present study reviewed the application of CGM in diabetic patients with different stages of CKD, including patients treated with hemodialysis, peritoneal dialysis or kidney transplantation. CGM provided a more precise and comprehensive estimation of mean glucose and glycemic variability, and had benefits in indicating acute episodes of hyper- or hypoglycemia. Therefore, the use of CGM is suggested as an alternative or complement to conventional glycemic indicators. Nevertheless, there is currently insufficient evidence to support the use of CGM in patients on dialysis or with kidney transplantation. Further clinical trials are required to improve and standardize its application for effective glycemic management and therapeutic regimen adjustment.

FOOTNOTES

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EDITORIAL

Pancreatic surgery and tertiary pancreatitis services warrant provision for support from a specialist diabetes team

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Abstract

Pancreatic surgery units undertake several complex operations, albeit with considerable morbidity and mortality, as is the case for the management of complicated acute pancreatitis or chronic pancreatitis. The centralisation of pancreatic surgery services, with the development of designated large-volume centres, has contributed to significantly improved outcomes. In this editorial, we discuss the complex associations between diabetes mellitus (DM) and pancreatic/periampullary disease in the context of pancreatic surgery and overall management of complex pancreatitis, highlighting the consequential needs and the indispensable role of specialist diabetes teams in support of tertiary pancreatic services. Type 3c pancreatogenic DM, refers to DM developing in the setting of exocrine pancreatic disease, and its identification and management can be challenging, while the glycaemic control of such patients may affect their course of treatment and

outcome. Adequate preoperative diabetes assessment is warranted to aid identification of patients who are likely to need commencement or escalation of glucose lowering therapy in the postoperative period. The incidence of new onset diabetes after pancreatic resection is widely variable in the literature, and depends on the type and extent of pancreatic resection, as is the case with pancreatic parenchymal loss in the context of severe pancreatitis. Early involvement of a specialist diabetes team is essential to ensure a holistic management. In the current era, large volume pancreatic surgery services commonly abide by the principles of enhanced recovery after surgery, with inclusion of provisions for optimisation of the perioperative glycaemic control, to improve outcomes. While various guidelines are available to aid perioperative management of DM, auditing and quality improvement platforms have highlighted deficiencies in the perioperative management of diabetic patients and areas of required improvement. The need for perioperative support of diabetic patients by specialist diabetes teams is uniformly underlined, a fact that becomes clearly more prominent at all different stages in the setting of pancreatic surgery and the management of complex pancreatitis. Therefore, pancreatic surgery and tertiary pancreatitis services must be designed with a provision for support from specialist diabetes teams. With the ongoing accumulation of evidence, it would be reasonable to consider the design of specific guidelines for the glycaemic management of these patients.

Key Words: Pancreatectomy; Pancreatoduodenectomy; Whipple's; Pancreatitis; Diabetes specialist; Type 3c pancreatogenic diabetes mellitus

Core Tip: In this editorial, we discuss the complex associations between diabetes mellitus and pancreatic/periampullary disease in the context of pancreatic surgery and overall management of complex pancreatitis, highlighting the consequential needs and the indispensable role of specialist diabetes teams in support of tertiary pancreatic services. In these settings, there is accumulating evidence that adequate glycaemic control at all stages improves outcomes, and that early involvement of specialist diabetes teams is of paramount importance to ensure a holistic management approach. The design of specific guidelines for the glycaemic management in these settings is warranted.

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INTRODUCTION

Pancreatic surgery units deliver a range of operations for benign, pre-malignant and malignant pancreatic and periampullary diseases[1]. These are notoriously complex operations, including the classic Whipple's pancreatoduodenectomy (PD) and its pylorus-preserving variation; distal pancreatectomy (DP) and its extended form called left pancreatectomy, with or without splenectomy; the more radical version of the latter, *i.e.* radical antegrade modular pancreatosplenectomy (RAMPS); central pancreatectomy; total pancreatectomy (TP) with or without splenectomy; tumour enucleations and other less common procedures[1,2]. In addition, operations undertaken for complications of acute pancreatitis or for chronic pancreatitis (CP) may involve various degrees of resections of pancreatic parenchyma and a number of different reconstruction techniques. Furthermore, pancreatitis itself may result in variable reduction of the functional pancreatic parenchyma. The aforementioned operations are associated with significant morbidity and mortality[1]. In recent decades, the centralisation of pancreatic surgery services with development of designated large-volume centres has gradually improved outcomes in conjunction with improvements in patient selection and operative techniques[1]. Characteristically, for the complex Whipple's operation, the perioperative mortality has fallen to less than 4.0% in high-volume centres, while postoperative morbidity remains high, up to 60%[1,3,4].

Diabetes mellitus and pancreatic disease

Diabetes mellitus (DM) may be associated with pancreatic and periampullary surgical diseases in different forms, particularly new-onset diabetes (NOD) as a potential presenting symptom of pancreatic ductal adenocarcinoma (PDAC)[5]. The most prevalent theory for the pathogenesis of the latter is that of a paraneoplastic manifestation induced by diabetogenic factors[6]. Type 3c pancreatogenic DM (T3cDM), refers to DM developing in the setting of exocrine pancreatic disease, including PDAC, CP, haemochromatosis, cystic fibrosis, and previous pancreatic surgery[7,8]. The entity remains underdiagnosed and underreported while its profile is characterised by low to normal fasting C-peptide, negative islet antibodies, coexistence with pancreatic exocrine insufficiency and often challenging glucose control with hyper- and hypoglycaemia, whilst episodes of ketoacidosis are rare. Whilst some individuals may be at least initially managed with oral agents, many will need insulin treatment[7-9]. T3cDM developing as a result of acute or CP is a heterogeneous entity

with variable clinical presentations, frequently misdiagnosed and treated as type 2 DM (T2DM)[10-12]. Meta-analyses from 2014 and 2019 found a rate of development of NOD ranging from 15% at 12 months following admission with acute pancreatitis, with a subsequent increase to 23% in following years[13-15]. Recent studies have shown an approximate 10% rate at 12 months[16,17]. Furthermore, many patients who require treatment for pancreatic or periampullary disease may have pre-existing DM[5]. In recent multicentre studies, approximately 20% of patients who underwent PD for malignancy had a preoperative diagnosis of DM[3,4]. Remarkably, up to 85% of patients with PDAC have hyperglycaemia or diabetes, while patients with NOD have a 5-8-fold higher risk of PDAC diagnosis within 1-3 years of developing DM[18]. Notably, recognition of NOD as a presenting symptom of PDAC is crucial for its early diagnosis, and distinguishing this form from T2DM has attracted significant interest in the last decade[6,7,18,19].

DM in the pancreatic surgery/pancreatitis setting

The group of patients with pancreatic/periampullary surgical disease and pre-existing prediabetes or DM requires special attention, as their glycaemic control may affect their course of treatment and outcome[20,21]. Furthermore, their diabetes management may be complex postoperatively and require intensification of treatment[18,19]. Notably, Tariq *et al* 's study of 216 patients undergoing DP, detected 40% of patients being preoperatively unaware of their dysglycaemic status (prediabetes or DM)[22]. Importantly, those with prediabetes were at increased risk of postoperative diabetes. Therefore, the authors concluded that adequate preoperative diabetes assessment is warranted for all patients ahead of pancreatic resection, to help identify those most likely to need initiation or escalation of glucose lowering therapy in the postoperative phase[22]. A higher rate of acute kidney injury[20] and postoperative pancreatic fistula (POPF) has been reported by some studies in diabetic patients undergoing pancreatic resection[20,21], while other studies have suggested absence of relationship[4] or even a possible protective effect of DM against POPF, owing to lower rates of high-risk pancreatic gland features[23,24]. Furthermore, as already mentioned, as a result of removal/loss of pancreatic parenchyma, new onset T3cDM may develop in the postoperative, as well as in the post-pancreatitis setting. It has been hypothesised that DP may confer a higher risk of postoperative development of diabetes compared to PD, as 70% of the β -cell mass appears to be located in the body and tail[22,25]. Tariq *et al*[22], in their study of 216 patients undergoing DP, found that 36% of non-diabetic and 57% of prediabetic patients developed DM at 2-year follow-up postoperatively[22]. Overall, the incidence of development of diabetes after pancreatic resection is widely variable in the literature, owing to different operative techniques, heterogeneous groups and the retrospective nature of most studies[22].

CP is a complex disease not infrequently requiring surgical treatment. The consensus statement of the International Study Group for Pancreatic Surgery on the standards for reporting on surgery for CP include the presence/absence of DM in the domain of "clinical baseline prior to surgery", and postoperative DM in the domain of "minimum outcome dataset"[26], using the terminology and reference ranges of the World Health Organization[27].

Another important group is represented by patients scheduled for TP with or without splenectomy, who will certainly have insulin-requiring diabetes postoperatively, regardless of their preoperative state[28]. These patients require specialist care prior to their surgery wherever possible, to ensure adequate education for the required insulin treatment and glucose monitoring. Early involvement of the specialist diabetes team is essential to ensure holistic management[9]. Specifically in the management of CP, the role of TP with islet autotransplantation (TPIAT) has been explored to avoid unstable postoperative diabetes. Consensus statements were based on strong agreement that, among other benefits, TPIAT offers glycaemic benefit over TP alone, and that other disease features as well as the islet mass transplanted may impact the outcomes[28].

As mentioned, a number of pancreatic diseases, including pancreatic tumours, CP and episodes of acute pancreatitis (especially necrotising)[14], may reduce the functional parenchyma of the gland and ultimately result in DM, either directly or by requiring surgical removal of part of the pancreas by the aforementioned different forms of pancreatectomy. The impact of these operations on the endocrine function of the pancreas depends on the extent, but also on the prior state of glycaemic control[9,29]. Importantly, it also needs to be noted that up to 35% of patients with pre-existing DM are reported to record improved glycaemic control after pancreatectomy[30,31].

Enhanced recovery after surgery concept and perioperative glycaemic control

In the current era of centralisation of services, which in the case of pancreatic surgery has evidently led to improved morbidity, mortality and oncological outcomes[1], large volume pancreatic surgery services worldwide commonly follow the principles of an enhanced recovery after surgery (ERAS) concept[32-34]. This is reflected in dedicated protocols, most of the time designed and approved locally, but largely following generally accepted principles in the field of pancreatic surgery. Despite possible variations, it is expected that pancreatic ERAS pathways include provisions for optimisation of the perioperative glycaemic control[32-34]. The most recent ERAS recommendations for PD from 2019, based on the best available evidence and on expert consensus, include postoperative glycaemic control among the standard parameters of the pathways[32]. They highlight that the available evidence supports an association between elevated blood glucose and adverse clinical outcomes, both in diabetic and non-diabetic patients. The optimal perioperative glycaemic target remains unclear, but in general, glucose levels should be kept as close to normal range as possible without causing hypoglycaemia. The level of evidence for the aforementioned recommendations is moderate, while the grade of recommendation is strong[32]. In forming these recommendations, a number of important facts have been taken into account. It has been noted that early hyperglycaemia (> 7.8 mmol/L), high glucose variability and high glucose values in the early period after PD, are significantly associated with development of complications[35,36]. A high preoperative glycated haemoglobin A1c level has been associated with almost a threefold increased risk of complications after surgery compared to normal levels[37]. A randomized controlled trial of patients undergoing liver and pancreatic surgery, including PD, whilst on intensive care, compared a group receiving perioperative intensive insulin therapy with a target blood glucose range of 4.4-6.1 mmol/L to an intermediate insulin therapy group with a blood glucose range of 7.7-10.0 mmol/L. The

intensive therapy group recorded lower rates of surgical site infection, POPF and shorter length of stay[38]. However, other multicenter trials in the intensive care setting, but not limited to post pancreatic surgery, have demonstrated that intensive insulin treatment results in increased incidence of hypoglycaemia and mortality compared to moderate glucose control[39-41]. The optimal levels of early postoperative blood glucose associated with improved clinical outcomes remain unclear[42], and notably no studies have examined glucose targets outside of the intensive care setting, where the challenges of maintaining tight glucose control are even higher.

A web-based survey undertaken through the ERAS® society and the International Hepato-Pancreato-Biliary Association membership was recently published and aimed to explore global awareness, perceptions and practice of ERAS for PD. Among 140 respondent surgeons, the majority rated highly the importance of postoperative glycaemic control (90%) as a component of the protocols[43]. Importantly, surgeons performing < 20 PDs per year were likely to face more significant challenges in implementing postoperative glycaemic control locally in the context of their enhanced recovery practice (P = 0.001)[43]. The guidelines of the Joint British Diabetes Societies (JBDS) for Inpatient Care Group from 2016 recommend that, for diabetic patients undergoing surgery, the principles of ERAS programmes should be followed[44].

Guidance, performance indicators and measured practice

As mentioned, the level of perioperative glycaemic control may have a direct impact on the course of recovery, length of stay and surgical outcomes. Ideally, to benchmark the quality of perioperative glycaemic control, appropriate available guidance and definitions can be used. In the United Kingdom, various guidelines are available that can be useful in aiding perioperative management of DM, such as those issued by JBDS, the Association of Surgeons of Great Britain and Ireland (ASGBI) and the Association of Anaesthetists of Great Britain and Ireland (AAGBI)[44-46]. The Healthcare Quality and Improvement Partnership (HQIP) commissioned the National Diabetes Inpatient Audit (NaDIA) which first took place in 2010 and followed an annual pattern. The NaDIA is based on information gathered by hospital staff about the quality of diabetes care provided to inpatients with DM during their hospital stay. For its purposes, a 'good diabetes day' was defined as any day in the management of a patient with DM when the number of tests per day followed the guidelines, there was no more than one blood glucose measurement of > 11 mmol/L and no measurement of < 4 mmol/L. The 2016 report, based on information collected from 209 acute hospitals in England and Wales, underlined that 28% of hospitals had no diabetes inpatient specialist nurses[47]. The ThinkGlucose campaign led by the National Health System (NHS) Institute for Innovation and Improvement expects close cooperation between the specialist diabetes team and hospital staff, for all hospitalised diabetic patients[48]. The NaDIA 2016 report found that 31% of people with DM who needed review by the diabetes team based on the ThinkGlucose criteria, did not meet this expectation[47], although notably this audit looked at all in-patients, not specifically those post pancreatic surgery. The National Institute for Health and Care Excellence (NICE) Quality Standard in England highlights that people with DM need access to a specialist diabetes team[47]. The NaDIA 2016 report underlined that hospitals should ensure that there are enough staff on the diabetes team to provide support in the delivery of safe diabetes care[47].

In 2018, the National Confidential Enquiry into Patient Outcome and Death (NCEPOD) published a report with wide multidisciplinary input, entitled "Highs and lows", after reviewing the quality of perioperative care provided to diabetic patients > 16 years old undergoing a surgical procedure in the United Kingdom (not confined to pancreatic surgery)[49]. This included assessment of patient care and service structure, at clinical and organisational level, respectively. Importantly, perioperative diabetes management was examined and several deficiencies were highlighted. A lack of clinical continuity across the different specialties in the perioperative pathway was noted and the absence of joint ownership and a joint multidisciplinary approach implied that DM management was falling between gaps in the surgical pathway[49], especially given that diabetes can be managed in primary care, community services or hospital-based specialist teams. Note was made of key diabetes team members being under-involved in patient management, including specialist diabetes nurses, pharmacists and dietitians[49]. Regular monitoring of blood glucose was underutilised at all phases of perioperative care[49]. The report emphasised that in 35.8% of diabetic patients in the study there was room for improvement in the clinical care, while 14.1% of cases required improvement in both clinical and organisational systems of care[49]. A number of areas for improvements and relevant recommendations were made[49]. Among those recommendations, the primary focus for action included the appointment of a Clinical Lead for Perioperative Diabetes Management. Among other tasks, they should lead at a local level on the writing and implementation of a policy for the multidisciplinary management of patients with DM who require surgery, in agreement with the guidelines of the JBDS [44,49]. Importantly, only 28% of hospitals were noted to have a named clinical lead for perioperative diabetes management. To ensure adequate assessment and optimisation of diabetes in view of upcoming elective surgery, the Clinical Lead would also be responsible for the appropriate utilisation of a standardised referral process, as well as for ensuring that diabetic patients undergoing surgery are safely handed over for close monitoring and adequate glycaemic control. The report also highlighted that for most of the patients whose diabetes was not managed by all the appropriate staff, early involvement of specialist diabetes nurses would have been valuable[49]. Additional recommendations included the development of a preoperative assessment clinic policy, as 43.4% of such clinics did not have a specific relevant policy, while where this existed, wider multidisciplinary involvement was variable. It was also recommended that the Clinical lead ensures that diabetic patients attending a preoperative assessment clinic have access to input from a specialist diabetes nurse and other members of the diabetes team, as required, and receive written guidance about the preoperative management of their DM. Moreover, it was recommended to avoid cancellations of elective surgical procedures in patients with DM, especially for known clinical issues, to locally audit such cancellations and to take appropriate action accordingly[49]. Diabetic patients should be prioritised on the operating lists to avoid prolonged starvation; 19.4% of patients appeared to have not been scheduled appropriately. Importantly, patients with diabetes should be provided with education and comprehensive information about their diabetes management at discharge from hospital as part of the discharge planning process, with the involvement of diabetes specialist nurses and the clinical lead

for perioperative diabetes management. In 20% of patients, adequate discharge arrangements for diabetes care were lacking[49]. Furthermore, largely in response to the findings of the NaDIA and the NCEPOD report, the Centre of Perioperative Care (CPOC) published in 2021 the "Guideline for perioperative care for people with diabetes mellitus undergoing elective and emergency surgery", consisting of a national joint standard and policy[50]. Understandably, whilst not all of these recommendations will be relevant to pancreatic surgery for malignancy, where it will rarely be appropriate to delay surgery to optimise metabolic control, they are important when elective surgery is planned for non-malignant pancreatic conditions. The CPOC guideline involved relevant recommendations across the wide spectrum of the perioperative pathway of diabetic patients undergoing elective and emergency surgery, including the referral, the stage before surgery, individualised plans and communication with healthcare teams, the time of admission, intraoperative management, ward management and time of discharge[50]. Notably, the recommended range of capillary blood glucose to maintain in the wards was set at 6-12 mmol/L[50].

Surgical and anaesthetic departments are expected to ensure morbidity and mortality meetings (MMM) for elective and emergency surgery, while the 2011 guidelines of the Royal College of Surgeons on emergency surgery recommend that regular departmental clinical audit and MMM should be undertaken and reported to the clinical governance committee[51]. In the NCEPOD study, only 25% of hospitals reported that an audit was performed on perioperative diabetes management[49].

Even though the NaDIA and the NCEPOD reports were not specifically focused on evaluating pancreatic surgical services and the provision of emergency pancreatitis surgery, their findings are of particular value to these services, given their particular challenges in perioperative diabetes care. However, there are a number of features of pancreatic surgery that mean specific guidelines would be helpful. This includes the very high prevalence of preoperative diabetes, the frequent use of insulin in Type 3c diabetes[13-17], and the fact that for patients who require a long hospital stay facing complications of pancreatitis or pancreatic surgery, the level of glycaemic control may have a further impact on the course and duration of recovery by affecting the course and management of complications.

Notably, nutritional assessment is essential in the preparation of diabetic patients for surgery as the reintroduction of nutrition postoperatively may be delayed and the disease process itself may result in dietary alterations. Glycaemic control is not uncommonly challenging in the postoperative period[48]. This is particularly applicable in patients undergoing pancreatic surgery. Furthermore, both patients undergoing pancreatic surgery and patients with severe pancreatitis may require short or long courses of Total Parenteral Nutrition. This may further complicate their glycaemic control which may become particularly challenging in this setting[52]. It is necessary that these groups of patients receive direct input from designated diabetes teams, with access to appropriate multi-disciplinary diabetes specialist clinicians, which may include specialist nurse or other practitioners, dieticians and diabetologists as required.

One aspect requiring special attention is the required education for patients undergoing pancreatic surgery or recovering from severe pancreatitis, who have or are likely to encounter changes of their initial glycaemic state, either in the form of new onset prediabetes/diabetes, or worsening of previous prediabetes/diabetes, and potentially a need for new forms of treatment.

There is clear agreement that patients undergoing pancreatic surgery should be evaluated by a diabetes team preoperatively and have sufficient insight into the management of their potential postoperative NOD[9]. Adequate follow-up with a diabetologist, a specialist diabetes nurse and a dietitian should be ensured in the outpatient setting, for appropriate education, optimisation of the glycaemic control and personalisation of insulin therapy[9]. The extent of pancreatic resection should be taken into account in planning the patient's diabetes education. For instance, in those undergoing a standard DP, the risk of development of DM is commonly uncertain, and needs to be assessed before, immediately after surgery, at discharge and subsequently during follow-up visits, since the glycaemic control may worsen or improve postoperatively[9]. People undergoing TP with or without splenectomy require intensive diabetes education, including dietary advice, and follow-up under a specialist diabetes team. Even though there is wide agreement on this matter, there is considerable disparity in the education patients receive preoperatively and postoperatively. Also, preoperative diabetes assessment is not practised uniformly[53]. Identifying preoperatively patients who are unwilling or incapable to monitor and maintain glycaemic control is crucial[9]. Notably, some authors advocate that inability to perform these tasks and lack of understanding on the part of the patient and/or family should be considered contraindications for TP[54]. Maker *et al*[54] noted significantly reduced postoperative morbidity and mortality following referral for preoperative patient education and subsequent surgical reassessment, to determine whether adequate understanding, support and resources were in place preoperatively. Furthermore, improved outcomes are recorded in the presence of follow-up diabetes education with a specialist team comprising diabetologist, diabetes nurse and dietitian. It is plausible that close collaboration between the patient and the diabetes specialists is essential to ensure the best possible care after pancreatic surgery, given the increased risk of hypoglycaemia in conjunction with the plethora of further potential issues to consider, including injection technique, self-monitoring of blood glucose, ketones, exercise, driving guidelines, travel, and alcohol intake[9]. Moreover, readmission rates are considerable for these patients, hence, further indicative of the requirement for additional input in the outpatient setting[9]. Consequently, discharge planning is essential for the holistic care of diabetic patients. Given the significant perioperative stress and dietary changes, these patients require close monitoring to ensure adequate glycaemic control in accordance with the recommended range of 6-12 mmol/L[49,50]. Inadequate discharge planning can cause readmissions for complications related to poor glycaemic control[49]. It is advised that ward staff work in close partnership with the DM specialists to ensure appropriate discharge criteria are met, and a collaborative process is in place. Patient education is fundamental to this process, as is the diabetes team that will follow up the patient[49].

CONCLUSION

Adequate glycaemic management is an essential aspect of the optimal provision of pancreatic surgery services and tertiary pancreatitis services, under the concept of centralisation and strive to meet standards of excellent care. Hence, the value of supporting these services with specialist input from diabetes teams is indispensable, and may become apparent at various stages of the clinical management. For patients undergoing elective pancreatic resection, this process starts at the preoperative stage with appropriate referral, evaluation and input, including education. This continues during the period of admission with the goal of optimising glycaemic control and providing adequate education, followed by regular post-discharge follow-up as required in each case. Equally, peri-admission and post-discharge specialist diabetes input should be made available in the complex management of patients with severe episodes of pancreatitis, as required. Adequate glycaemic control at all stages can clearly impact outcomes and is therefore essential. As such, it is plausible to consider that pancreatic surgery services and tertiary pancreatitis services must be designed with a provision for support from specialist diabetes teams and that close working between surgical and diabetes teams is essential. As evidence is accumulating, it would be reasonable to consider the design of specific guidelines for the glycaemic management of these patients.

FOOTNOTES

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REVIEW

Role of renin-angiotensin system/angiotensin converting enzyme-2 mechanism and enhanced COVID-19 susceptibility in type 2 diabetes mellitus

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Abstract

Coronavirus disease 2019 (COVID-19) is a disease that caused a global pandemic and is caused by infection of severe acute respiratory syndrome coronavirus 2 virus. It has affected over 768 million people worldwide, resulting in approximately 6900000 deaths. High-risk groups, identified by the Centers for Disease Control and Prevention, include individuals with conditions like type 2 diabetes mellitus (T2DM), obesity, chronic lung disease, serious heart conditions, and chronic kidney disease. Research indicates that those with T2DM face a heightened susceptibility to COVID-19 and increased mortality compared to non-diabetic individuals. Examining the renin-angiotensin system (RAS), a vital regulator of blood pressure and pulmonary stability, reveals the significance of the angiotensin-converting enzyme (ACE) and ACE2 enzymes. ACE converts angiotensin-I to the vasoconstrictor angiotensin-II, while ACE2 counters this by converting angiotensin-II to angiotensin 1-7, a vasodilator. Reduced ACE2 expression, common in diabetes, intensifies RAS activity, contributing to conditions like inflammation and fibrosis. Although ACE inhibitors and angiotensin receptor blockers can be therapeutically beneficial by increasing ACE2 levels, concerns arise regarding the potential elevation of ACE2 receptors on cell membranes, potentially facilitating COVID-19 entry. This review explored the role of the RAS/ACE2 mechanism in amplifying severe acute respiratory syndrome coronavirus 2 infection and associated complications in T2DM. Potential treatment strategies, including recombinant human ACE2 therapy, broad-spectrum antiviral

drugs, and epigenetic signature detection, are discussed as promising avenues in the battle against this pandemic.

Key Words: Angiotensin-converting enzyme 2; Angiotensin-converting enzyme inhibitors; Angiotensin-II receptor blockers; Complex diseases; COVID-19; Type 2 diabetes

Core Tip: This manuscript explored the complex connection between type 2 diabetes mellitus and coronavirus disease 2019 (COVID-19), emphasizing the heightened susceptibility of diabetic individuals. It revealed the crucial involvement of the renin-angiotensin system and angiotensin converting enzyme (ACE)/ACE2 enzymes, clarifying how decreased ACE2 expression in diabetes amplifies renin-angiotensis system activity, contributing to inflammation and fibrosis. While ACE inhibitors exhibit therapeutic potential, concerns arise regarding their potential to facilitate viral entry. The review suggested that innovative approaches, including recombinant human ACE2 therapy and the detection of epigenetic signatures, present promising avenues for addressing COVID-19 and its complications in type 2 diabetes mellitus.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by a novel virus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which globally expanded from Wuhan, China at the end of 2019. The World Health Organization declared COVID-19 a global pandemic on March 11, 2020 (World Health Organization Situation Reports 51). According to weekly epidemiological updates globally, over 4500 deaths and more than 836000 new COVID-19 cases had been recorded from June 19 to July 16, 2023. More than 6.9 million deaths and 768 million confirmed cases were recorded worldwide[1]. Most severe COVID-19 cases have been observed in elderly people or comorbid individuals, including type 2 diabetes mellitus (T2DM), hypertension, cardiovascular diseases (CVDs), chronic renal and lung disorders, and cancer[2-5]. According to a meta-analysis including 1527 patients in China, patients with diabetes or hypertension had a two-fold increase in risk of severe COVID-19 or requiring intensive care unit (ICU) admission, while it increased to three-fold in patients with cerebrovascular disease[6]. Several studies have shown that SARS-CoV-2 along with pneumonia, may be detrimental to the other body organs including the heart, liver, and kidneys[7,8]. In addition to this, a high prevalence of comorbidities was also reported in COVID-19 patients[4,9-12]. Therefore, the treatment of comorbidities should be given more att-ention, especially in elderly patients with severe underlying conditions.

To effectively manage patients with comorbid disorders, it is crucial to comprehend the relationship between COVID-19 and chronic diseases. Additionally, it enables medical professionals to lessen the side effects linked to this disease. Hence, understanding these mechanisms will help develop a potential treatment for COVID-19. Herein, this review offered proof of the severe COVID-19 manifestation in T2DM and other chronic illnesses, including heart disease, hypertension, and myocardial infarction. More importantly, it analyzed the specific management techniques for patients with the aforementioned comorbid disorders to obtain the greatest therapeutic outcome, described in detail the molecular pathways through which COVID-19 causes these diseases, and highlighted the importance of these strategies.

OVERVIEW OF THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM AND INTRODUCTION TO ANGIOTENSIN CONVERTING ENZYME 2

Angiotensin converting enzyme 2 (ACE2) is a key enzyme in the renin-angiotensin-aldosterone system (RAAS), which plays a critical role in the regulation of several physiological processes in the human body. ACE2, a component of RAAS, oversees vasoconstriction to manage blood pressure, electrolyte and fluid balance, and renal salt reabsorption[13]. In response to diminished renal blood flow, juxtaglomerular cells convert prorenin to renin, which is then released into the circulation spontaneously. Plasma renin converts hepatic angiotensinogen to angiotensin I (Ang-I), and Ang-I is further transformed into the vasoconstrictor Ang-II by ACE (Figure 1). By converting Ang-II into Ang-(1-7), a vasodilator, ACE2 is essential for maintaining system balance[13]. Thus, ACE2 controls several pathological states, including oxidative stress, fibrosis, vasoconstriction, and inflammation (Figure 1). To mitigate RAAS hyperactivity, specific medications disrupt this system through various approaches. For instance, to reduce the synthesis of robust Ang-II, ACE enzyme inhibitors (ACEi) are commonly employed. Another method involves the use of angiotensin-II receptor blockers (ARBs) to inhibit angiotensin-II type 1 receptor (AT1R), thereby controlling an overactive RAAS[14].

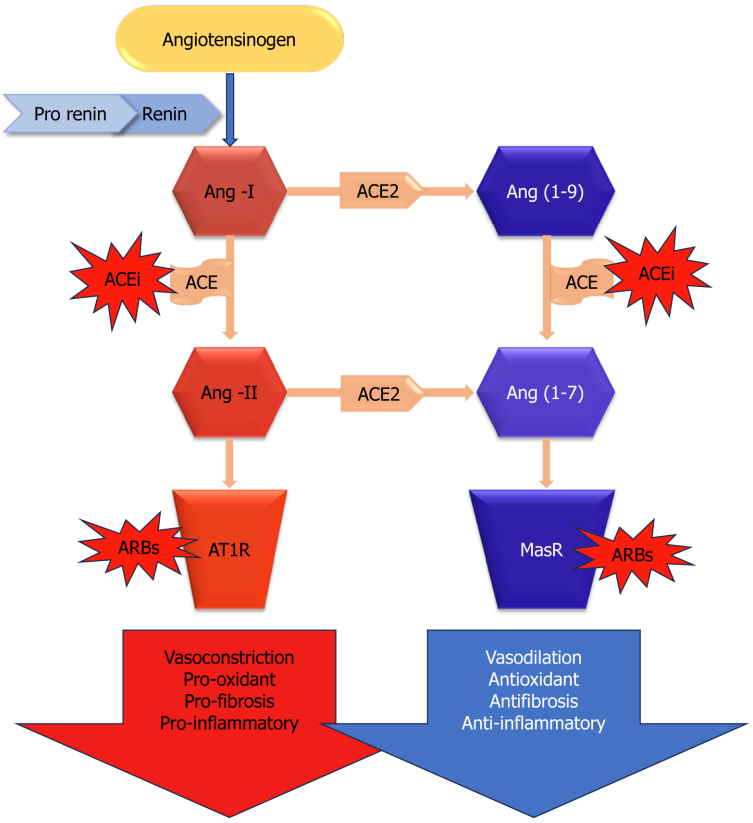

Figure 1 Renin angiotensin aldosterone system: Its components and workflow. ACE: Angiotensin-converting enzyme; ACE2: Angiotensin-converting enzyme 2; ACEi: Angiotensin-converting enzyme inhibitor; Ang(1-7): Angiotensin 1-7; Ang(1-9): Angiotensin 1-9; Ang-I: Angiotensin I; Ang-II: Angiotensin II; ARBs: Angiotensin-II receptor blockers; AT1R: Angiotensin-II type 1 receptor; MasR: MasR receptor.

Role of renin-angiotensin system, ACE, and ACE2 in diabetes susceptibility

Additionally seen in the glomerulus, ACE2 colocalizes with ACE on the apical surface of the proximal tubules[15]. Similar to ACE in distribution, ACE2 was shown to be confined to pancreatic acini and islets[16]. It has been shown that in diabetic animal models, ACE2 pharmacological suppression[15,17] and genetic ablation[18] both exacerbate albuminuria and the associated glomerular lesions. Additionally, diabetic nephropathy patients' kidney biopsies[19] and diabetic rodent models[15] have demonstrated decreased glomerular expression of ACE2. Thus, in order to address renal impairment linked to diabetes, increasing the activity of this enzyme by therapeutic targeting of ACE2 has been suggested[15,20].

Changes in glucose tolerance and decreased first-phase insulin production have been seen in *ACE2* knockout mice, indicating that diabetes development may be influenced by ACE2[21]. According to reports, pancreatic β cells contain collectrin, another ACE homolog, which may play a role in insulin production and β cell proliferation despite its unknown function[22]. Concentrating on comprehending the involvement of ACE2 in diabetes, emphasizing ACE2 within the kidney could emerge as a crucial focal point for addressing and preventing diabetic kidney disease. This progressive condition is marked by thickening and expansion of the mesangial matrix around the glomerular basement membrane, the presence of elevated protein in urine, and associated features linked to kidney disease. In recent times, several research studies have also suggested that the loss of podocytes[23-27] is connected to the deterioration of glomerulopathy[28]. The kidney exhibits a completely operational local renin-angiotensin system (RAS), with the ability to produce Ang-II in hyperglycemic circumstances[29,30] as observed by its activation[31].

The presence of ACE is reported in the kidney, pancreas, adipose tissue, and liver. Its expression level is elevated in the endocrine system involving the pancreas in diabetes and in the early stages of diabetic nephropathy. The proposition suggests that ACE2 plays an equal role in compensating for both diabetic nephropathy and diabetes. Furthermore, it appears that mice lacking ACE2 also had higher blood glucose levels following fasting, as evidenced by their elevated fasting blood glucose levels. A novel method for examining the role of ACE2 in diabetes research may be found in mice [32]. On the apical surface of the proximal tubules and in the glomerulus, ACE2 colocalizes with ACE[15]. Similar to ACE, ACE2 was shown to be distributed in the pancreas and localized to acini and islets[16].

More than 20 years ago, Chappell *et al*[33] discovered that the pancreas, like the kidney, expresses significant components of a local angiotensin peptide-producing machinery. With decreased levels of Ang-(1-7) and undetectable quantities of Ang-I, the investigators found that Ang-II was the most prevalent angiotensin peptide in the pancreas. As it has been speculated to do in the kidneys and other organs, Ang-(1-7) may mitigate the effects of Ang-II in the pancreas [34]. In comparison to the plasma, both peptides were found in much larger concentrations in the pancreas[33]. Ang-II has the ability to inhibit blood flow in a dose-dependent manner and delay the generation of insulin in the Langerhans islets, as revealed by Carlsson *et al*[34]. The effects of AngII are consistent with increasing blood flow by RAS blockage

with ACE inhibitors or AngII receptor antagonists[34]. Moreover, it has been demonstrated that these medications lessen pancreatic fibrosis and inflammation[35]. In a noteworthy study, Tikellis *et al*[16] found that in the Zucker diabetic fatty rat, RAS inhibition enhanced structural parameters in relation to increased first-phase insulin secretion and reduced islet fibrosis. Given clinical evidence that RAAS suppression may be associated with a decreased risk of T2D with a new start, these findings are particularly noteworthy[36]. Though more recent studies have cast doubt on these results[37], ongoing current investigations have demonstrated that RAAS blockage aids in the prevention of T2DM.

Based on animal research, it was hypothesized that ACE/ARB therapy increases ACE2 expression. After the recognition of ACE2 as the receptor for SARS-CoV-2[5,38], there is apprehension that an excessive expression of ACE2, resulting in a faster uptake of the virus, might worsen lung damage and elevate the risk of a fatal outcome in individuals with COVID-19. Current clinical analyses[39] and assessments[40] regarding the COVID-19 pandemic raised these concerns without thoroughly examining data from studies involving both animals and humans, resulting in prompt conclusions and even instilling fear among physicians and patients utilizing ACEis or ARBs. There are current investigations aimed especially at demonstrating that RAS blockage aids in the prevention of T2DM.

ACE2 shedding and RAAS overactivity: Implications for glycemic homeostasis

Over time, expression of ACE2 and functioning in several tissues decrease in complications such as T2DM and hypertension[19,41-43]. The mechanisms behind the reported decrease in ACE2 in these illnesses are unclear. One explanation for declining levels of ACE2 might be its depletion from tissues. Cell-membrane-bound ACE2 has been discovered to be cleaved by a sheddase, disintegrin, and metalloproteinase (ADAM17) or tumor necrosis factor-α (TNF-α) converting enzyme[44], releasing some of the tissue-resident protein into the bloodstream. In fact, ADAM17 may be the cause of ACE2 shedding in T2DM patients due to its elevation in human carotid atherosclerotic plaques[45]. Interestingly, the pancreas, brain, adipose tissues, and vascular smooth muscle cells all express more ADAM17 when exposed to Ang-II [46,47]. The phenomenon of ACE2 downregulation in response to RAS overactivity in the islets of mice can be attributed to ACE2 shedding mediated by ADAM17[42]. The surge in ADAM17 expression is lowered by ACE2 treatment, suggesting that levels of Ang-II can affect levels of ADAM17[42]. In summary, preliminary data points to a possible link between RAS hyperactivity and decreased glycemia; restoring lost ACE2 in the islets seems to control activity of RAS and hence raise glycemia. Restoring lost ACE2 seems to lower RAS activity, which in turn lowers blood sugar levels. To corroborate these early findings, more research into the connection between ACE2 shedding and glycemia is needed.

Role of ACE and ACE2 in COVID-19 susceptibility

SARS-CoV-2 belongs to the Beta-coronavirus genus, which encompasses other notably contagious viruses including the Middle East respiratory syndrome coronavirus and the SARS coronavirus (SARS-CoV)[48]. SARS-CoV and SARS-CoV2 have about 80% sequence similarity[49]. Both viruses enter cells by binding to the ACE2 with their spike proteins (S-protein)[49]. An extraordinarily high rate of human transmission makes SARS-CoV-2 different[50]. Indeed, SARS-CoV-2 exhibits a heightened attraction to ACE2, contributing to its increased transmissibility. Through its S-protein, SARS-CoV-2 binds to human cells. ACE2[51], which is widely expressed on the surfaces of pulmonary alveolar epithelial cells, venous and arterial endothelial cells, arterial smooth muscle cells, and small intestine enterocytes, is one of the main SARS-CoV-2 receptors[38,52-55].

The S-protein is split into S1 and S2 subunits after viral infection[54]. While the S2 subunit aids in membrane fusion, the S1 subunit directly interacts with the host cell's ACE2 receptor through its receptor binding domain[54]. SARS-CoV-2 and SARS-CoV have very similar receptor binding regions. In contrast, the SARS-CoV-2 binding site exhibits enhanced stability and compactness, displaying a greater affinity for ACE2 and its ability to infect the host cell is increased by the presence of a Furin at the cleavage site[56,57]. After the binding is complete, ACE2 activity is downregulated[54]. ACE2 downregulation is due to the activation of ADAM-17/TNF-α converting enzyme by the SARS S-protein, which cleaves and releases ACE2[44,58]. Increased circulating levels of ACE2 were found interconnected with the severity of lung injury and the viral load[56]. The downregulation of ACE2 after SARS-CoV-2 infection increases Ang-II concentration in the lungs causing severe lung injury. Another study reported ACE2 downregulation in the heart and severe heart injury[59].

There are a number of potential causes for the increased severity of COVID-19 in individuals with concomitant illnesses. The increased expression of ACE2 in certain organs, including the kidneys, small intestine, lungs, and heart[52,56,60], might elucidate the severe manifestation of COVID-19 in specific patient groups. This is because ACE2 serves as the functional receptor through which SARS-CoV-2 enters cells[61]. In addition, the overactive immune response to the virus that contributes significantly to the disease severity is known as the "cytokine storm"[62,63]. It is intriguing to note that COVID-19 was shown to trigger the abrupt beginning of long-term organ damage in people who did not already have chronic illnesses. One of these consequences is cardiac injury[64], along with kidney injury[65], renal injury[65], liver damage[67], gastrointestinal disorders[66], and acute or chronic diabetes[68]. This makes COVID-19 more challenging and increases the likelihood that many infected people may develop chronic morbidities that are incapacitating. Interestingly, there is a scarcity of clinical data that supports these pathophysiological processes.

Mechanism of SARS-CoV2 entry into cells

Typically, coronaviruses require two cleavage events of the S-protein for their entry process (Figure 2). Two cleaves occur: One at the S1-S2 subunit junction and the other at the S2′ location inside the S2 subunit[69]. Regarding SARS-CoV-2, the polybasic sequence cleaves at the S1-S2 border as the virus matures in an infected cell. Nevertheless, once ACE2 binds, the target cell cleaves at the S2′ location. The S2′ cleavage site in the S2 subunit is made visible by conformational changes in the S1 subunit caused by the interaction between the virus and ACE2. The particular proteases that cause the cleavage at the S2′ site differ according to the SARS-CoV-2 entry pathway that is selected.

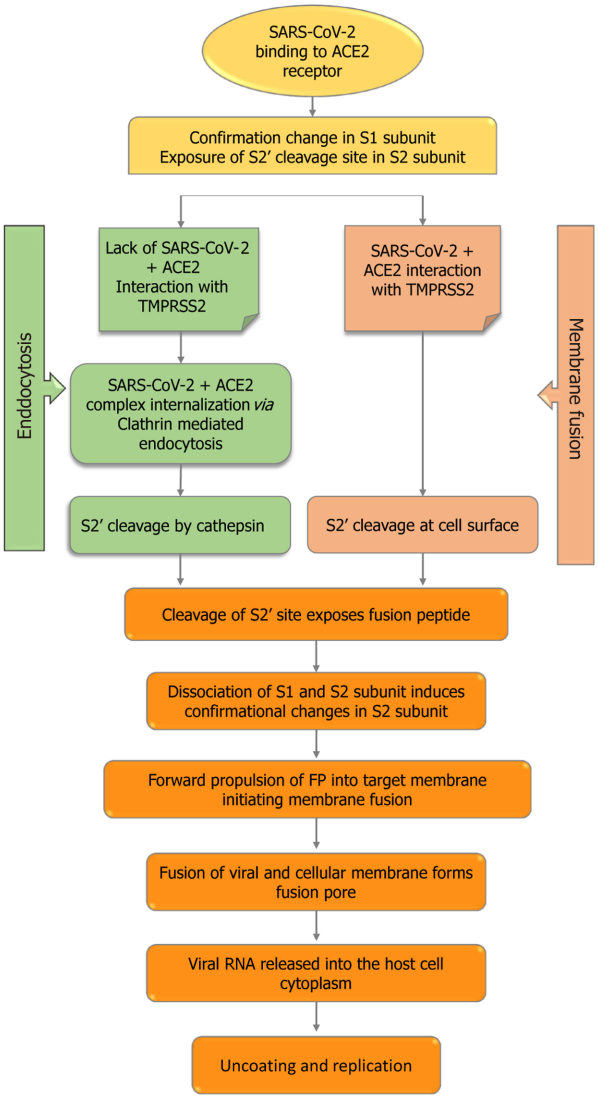

Figure 2 Mechanisms of how the virus enters cells *via* the angiotensin-converting enzyme 2 receptor. ACE2: Angiotensin converting enzyme 2; FP: Fusion peptide; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; TMPRSS2: Transmembrane protease serine 2.

When transmembrane protease, serine 2 (TMPRSS2) is insufficient in the target cell or when a virus-ACE2 complex is not in contact with TMPRSS2, the virus-ACE2 complex is internalized *via* clathrin-mediated endocytosis[70]. Cathepsins within endolysosomes cleave the S2′ site as a result of this internalization, and they require an acidic environment to function. Conversely, in the presence of TMPRSS2, S2′ cleaves at the cell surface. The fusion peptide is exposed in both routes by the cleavage of the S2′ site, and the S2 subunit undergoes major conformational changes as a result of S2 splitting from S1, especially in heptad repeat 1[69]. This starts the process of membrane fusion by pushing the fusion peptide into the target membrane. Viral RNA is released into the host cell cytoplasm for uncoating and replication through a fusion hole created when the viral and cellular membranes fuse[69].

Impact of RAAS expression on T2DM patients suffering from COVID-19

Increased blood glucose and glycosylation product synthesis in patients with diabetes could enhance the working of RAAS, notably Ang-II. When exposed to excessive amounts of Ang-II, a variety of tissues can stimulate dihydro nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and increase oxidative stress, which can lead to increased insulin resistance, complications from diabetes, and a worse prognosis for COVID-19[71-73]. Moreover, Ang-II worsens diabetes by promoting fibrosis, cell death, and a reduction in the islet blood supply and early stages of insulin synthesis[73]. Ang-I is converted to Ang-(1-9) by ACE2, whereas Ang-II is converted to Ang-(1-7). These two physiologically active downstream peptides encourage vasodilation, which has been linked to anti-inflammatory, antifibrotic, and antiproliferative properties[74]. In addition to partially protecting islet cells and preventing diabetes, ACE2 suppresses conventional RAAS activation[75]. These receptors are downregulated as a result of the interaction of SARS-CoV-2 and ACE2, which is followed by membrane fusion and viral entry into the cell. This exacerbates cellular damage, hyperinflammation, and respiratory failure[76,77]. Increased ACE2 insufficiency may exacerbate the dysregulation between the "protective" ACE2 Ang-(1-7) Mas receptor axis and the "adverse" ACE Ang-II AT1R axis following viral invasion[77]. Insulin secretion may be hampered by ACE2 downregulation upon viral entry if Ang-II is not inhibited. These factors

may play a role in the rapid degradation of pancreatic cell function and the development of diabetic ketoacidosis[78], suggesting that the prognosis for patients with diabetes after SARS-CoV-2 infection may be bad. Nevertheless, it is uncertain if ACE2 has a mechanistic role in the development of dysglycemia or elevated complications in diabetics[79]. Evidence suggests that RAS inhibitors, which include ACEi and ARBs, increase the expression of ACE2, which may have a protective effect against diabetes and hypertension but also make it easier for viruses to enter host cells, which may increase the likelihood or severity of infection[80]. However, recent studies have not demonstrated that using ACEi or ARBs raises the likelihood of SARS-CoV-2 infection or the risk of serious consequences in COVID-19 individuals[74,81-83].

LINK BETWEEN DIABETES, COVID-19, AND ACE2: A CLOSER LOOK

Diabetes is considered to be one of the leading causes of death worldwide. Elderly people with T2DM are more severely prone to infections such as pneumonia and influenza[84,85]. The International Diabetes Federation estimated that 537 million adult individuals were diagnosed with diabetes in 2021[86], with this figure anticipated to climb to 643 million by 2030 and 783 million by 2045[87].

Several studies could not find a clear association between COVID-19 severity and diabetes[12,88]. However, studies in Italy[89] and China[2] showed the perilous threat of mortality and severe COVID-19 in older patients with some chronic diseases, including diabetes. Diabetes has been identified as an elevated risk for severe COVID-19 since the pandemic began. It is indeed one of the most frequently documented comorbidities among COVID-19 patients who were brought to the ICU and later passed away[3,11]. In a study that included 52 COVID-19 patients who were brought to the ICU, 32 (61%) of the patients had passed away 28 d after discharge; the most prevalent comorbidities in these patients were diabetes (22%) and cerebrovascular illnesses (22%)[4].

A different study involving 44672 individuals with SARS-COV-2 found that the COVID-19 case fatality rate was 7.3% in diabetic individuals and 2.3% in non-diabetic individuals[3]. Numerous variables may be responsible for the severe presentation, even if the cause of the poor prognosis in patients with diabetes is unknown[90]. First of all, poorly managed diabetes compromises the ability of the immune system to fight viral infections[91]. The capacity to eliminate viruses is particularly hampered by poor T cell function, which weakens the natural defense system[92]. Second, plasminogen levels are increased in diabetic individuals[93]. This particular protein cleaves the S-protein of SARS-CoV-2, which enhances the viral cellular entry; further, it leads to an increase in the viral contagion and infestation of the virus [93]. Alongside, inflammatory biomarkers like interleukin-6 (IL-6), C-reactive protein, and D-dimer were found to have increased levels in diabetic SARS-COV-2 individuals in comparison to those without diabetes, which indicates that the risk of worsening COVID-19 outcomes might be increased due to T2DM[64].

Due to their renal protective properties, ACEi is frequently prescribed to diabetic patients. This class of drugs raises the expression of ACE2, due to which viral entrance could be facilitated into the cells of the host[94]. *FURIN* and *TMPRSS* are two additional genes linked with cellular entry of SARS-CoV-2 that remain upregulated in T2DM, according to microarray-based transcriptome profiling for the disease[95]. Finally, patients with diabetes having additional ailments such as coronary artery disease, chronic renal disease, and hypertension have a substantially poorer COVID-19 outcome[90]. COVID-19 puts patients with diabetes at a higher risk of serious consequences. Diabetes, as previously noted, is regarded as one of the most frequent comorbidities in this pandemic. As a result, greater effort should be expended to comprehend the molecular mechanisms behind this danger.

Data are scarce regarding the advancement of diabetic complications and glucose metabolism in individuals having COVID-19. According to retrospective research, 10% of patients with COVID-19 and T2DM had at least one episode of hypoglycemia (< 3.9 mmol/L)[5]. However, SARS-CoV-2 infection in diabetic individuals may result in elevated stress levels and the release of more hyperglycemic hormones, such as glucocorticoids and catecholamines, which raises blood sugar levels[96].

Possible mechanisms for SARS-CoV-2 infection in patients with diabetes

Numerous vascular and metabolic problems associated with diabetes might impact our body's ability to respond to infections[97]. Hyperglycemia as well as insulin resistance enhance the synthesis of adhesion molecules, which promote inflammation of tissues, and increase the production of proinflammatory cytokines, advanced glycation end products, and oxidative stress[97,98]. The undivulged phenomena that increases the risk of infestation in patients with diabetes may be this inflammatory response[97].

Some studies have reported an association of hyperglycemia with defects in immunity[99]. Compliment activation dysfunction[100] and abnormal delayed-type hypersensitivity reaction[99] have been reported in diabetic individuals. Studies conducted *in vitro* revealed that exposure to elevated concentrations of glucose enhanced influenza virus infection and replication in pulmonary epithelial cells, suggesting elevated viral replication *in vivo* during hyperglycemic circumstances[101].

COVID-19 epidemiology and its relationship to cardiometabolic diseases are poorly known. Current statistics reveal that people with COVID-19 and diabetes mellitus have a higher risk of death in comparison with individuals without diabetes mellitus. Resistance to insulin is caused by COVID-19 in individuals, resulting in persistent metabolic problems that did not exist before infection. Patients with diabetes are more vulnerable to SARS-CoV-2 infection than individuals who do not have diabetes. With infection, ACE2 expression declines, magnifying Ang-II activity and leading to insulin resistance, an increased immunological response, and severe SARS-COV-2 infection[102]. Exocrine and endocrine pancreatic synthesis of ACE2 is likely to be associated with an increased presentation of diabetes in subgroups of

critically unwell SARS-CoV-2 infected persons[79]. Through binding to the ACE2 receptor as seen in SARS-Cov-1[103], SARS-CoV-2 promotes pancreatic islet destruction and the development of acute diabetes[4].

ACE2 is one of the main receptors of both SARS-CoV-2[51] and SARS-CoV[104]. When CoVs get introduced to host cells, the CoVs attach to specific ACE2 receptors with the help of their S-proteins. The S-protein is then cleaved by host cell protease, allowing the virus to enter and multiply within the host cells[51]. Patients with diabetes and hypertension frequently use medications called ACEi and ARBs[105]. These drugs are known to increase the ACE2 concentration, and thus their use is controversial as it might negatively affect the outcomes of COVID-19 patients[55]. On the other hand, some recommended ARBS and ACEi might be beneficial[106].

COVID-19 VULNERABILITY: UNRAVELLING THE ROLE OF COMORBIDITIES

SARS-CoV-2 infection has been potentiated by primary complications that are illustrated as T2DM and its associated metabolic comorbidities. Mentioned below are the other related comorbidities and their possible mechanisms involved in the pathophysiology of SARS-CoV-2 infection and different strategies involved in the treatment of COVID-19.

Hypertension and COVID-19

The initial study in China indicated a high occurrence (30%) of hypertension in severe SARS-COV-2 cases[107], which is not very surprising as 23.2% of the adult population of China are estimated to have hypertension[108]. According to another report, out of 406 patients who died from COVID-19, 39.7% of patients had hypertension[109], which is higher than the general population. Hypertension may also result in other cardiovascular risk factors like hypertension-mediated organ damage, diabetes, or cardiovascular complications[110], and their prevalence increases with age.

Italy was the first European country that was most severely affected by COVID-19[111,112]. According to a report published on June 18, 2020 by *Epidemiology of Public Health*, the median age of patients in Italy who died with COVID-19 was 82 years, and 66.8% deceased COVID-19 patients had pre-existing hypertension (https://www.epicentro.iss.it/en/coronavirus/sars-cov-2-analysis-of-deaths). These numbers were much higher than reported in China but were consistent with estimated high prevalence of hypertension of this age group in Italy[113]. Furthermore, 17% and 30% of deceased patients with COVID-19 were being treated with ARBs and ACEi, respectively. These data suggest that COVID-19 patients with hypertension exhibit an increased fatality rate as compared with overall infected patients, with elderly patients being at greatest risk.

Possible mechanisms for SARS-CoV-2 infection in hypertension patients

A recent study conducted to understand the role of immune dysregulation in hypertension[114] provided the possible mechanism linking immune dysregulation and COVID-19 severity. An increased level of systemic IL-7, IL-6, IL-2, C-X-C motif chemokine 10, chemokine ligand 2, granulocyte colony-stimulating factor, and TNF-α was observed in COVID-19 patients[7]. Interestingly, these are the same cytokines that are associated with hypertension development in interventional[114], clinical observational[115], and experimental studies[116,117].

Another study reported dysregulation of CD8+ and CD4+ cells in hypertension[118], indicating elevated productivity of proinflammatory cytokines, which includes those related with COVID-19 (IL-6, IL-7, IL-17, TNF-α, and interferon-γ) [119]. Hypertension was associated with a distinct immune senescent profile of CD8+ cells[114,119,120], which were likely to overproduce cytokines but are weak in antiviral defense. These mechanisms significantly contribute to accelerated end-organ damage[121,122]. All these studies somewhat demonstrate the association of hypertension with the severity of COVID-19. In order to test this hypothesis, observational studies are recommended on a large scale to analyze the relationship between hypertension and COVID-19.

ARBs are usually used for the treatment of hypertension and are considered the main therapy for hypertension and associated renal and cardiovascular comorbidities[110]. Several reports showed upregulation of ACE2 upon treatment with ACEi and/or ARBs[123]. Hence, some authors hypothesized that RAAS blockers may enhance the susceptibility and COVID-19 risk. They recommended avoiding ARBs and ACEi during the COVID-19 pandemic[40,124]. Several studies conducted to investigate the effect of RAS blockade on ACE2 showed variable results. These variations may depend on different pathological conditions, like hypertension *vs* normotension, animal strains used, organs investigated, and most importantly type and dosage of the drug. Moreover, very little human research has been conducted to look at how RAS blockage affects the expression of ACE2. A study showed that olmesartan, an ARB, can significantly increase urinary ACE2 in hypertensive patients when treated for > 1 year[125]. Similar results were seen in patients with diabetic nephropathy upon treatment with olmesartan for 24 wk[126]. On the other hand, urinary ACE2 levels were not affected by ACEi or ARBs in diabetic patients[127].

There is no evidence that shows the usage of ACEi and/or ARBs that directly enhances the susceptible SARS-CoV-2 and its severity and infectivity. Therefore, it is recommended to continue the use of RAAS blockers in stable COVID-19 patients with hypertension and other related diseases, like T2DM, heart failure, and chronic kidney disease[110,128].

CVDs and COVID-19

Several studies of COVID-19 showed extreme variability ranging from asymptomatic to some mild symptoms like dry cough, fatigue, and fever. According to some early reports, a high prevalence and association of comorbidities was seen with severe COVID-19 and mortality[2,4,5,7,10,12,82]. Among different comorbidities, the involvement of heart-related ailments appeared more significant. In a report from the Chinese Control for Disease Control and Prevention of 72314 cases, 1023 deaths were reported among 44672 confirmed cases (*i.e.* 2.3% mortality rate). However, this percentage went

up to 10.5% in instances of diabetes, 7.3% in cases of cardiovascular illness, 6.3% in situations of chronic respiratory conditions, and 6.0% in cases of hypertension[3].

Myocardial damage during COVID-19 might be asymptomatic, similar to other acute diseases. It can only be identified by detecting troponin levels beyond the 99th percentile upper reference range. Patients with CVDs were shown to be particularly affected by COVID-19[2], and many of them had myocardial stress[9], active myocardial damage[5,9,129], and cardiomyopathy[9] while they were unwell. There was an increase in the cardiac troponin levels in 8%-12% of unselected COVID-19 patients[5,7,64,129]. Other studies also reported higher plasma levels of N-terminal pro-brain natriuretic peptide in cases with myocardial injury, although these increased levels were not independently associated with the outcomes[64,129].

Some of the cardiac complications, like cardiomegaly, hypotension, and heart failure were previously seen in SARS-CoV infections[130]. Acute myocarditis or myocardial damage may lead to severe cardiac dysfunction or heart failure in COVID-19 cases. This is often difficult to diagnose and controversial. Although very few COVID-19-associated acute myocarditis cases were reported[131-134], they might be severe with low cardiac output and hypotension that needed end-stage heart failure inotropic therapy. The endomyocardial biopsy showed limited or no myocardial necrosis and different levels of myocardial inflammation[131-134].

Only 1 of the 2 patients who underwent endomyocardial biopsy met the criteria for acute myocarditis[133]. SARS-CoV-2 was also detected in macrophages but not in cardiomyocytes in a different instance. The biopsy results from these cells revealed only modest interstitial cardiac inflammation and some non-specific alterations in heart myocytes, such as lipid droplets and myofibrillar lysis[134]. These data indicate that the virus does not have any direct pathogenetic role, even though it can reside within the heart[5,135]. Hence, it can be assumed that apart from viral infection, other mechanisms responsible for myocardial injury may exist[5,135,136].

Possible mechanisms for SARS-CoV-2 infection in patients with CVDs

Cardiomyocytes, coronary endothelial cells, and cardiac fibroblasts all have ACE2 on their surface. It has been discovered that individuals with diabetes, cardiovascular conditions, and those using ARBs and/or ACEi had elevated levels of ACE2[136-140]. This has been reported in myocardium tissue samples of patients with end-stage heart failure[139,140], experimental models[137,138], and circulating plasma levels of ACE2[140]. *ACE2* knockout mice develop heart failure with reduced ejection fraction and systolic dysfunction in left ventricle[59]. Several studies conducted on experimental models showed that *ACE2* gene overexpression reduced fibrosis, oxidative stress, and myocardial hypertrophy, which improved the diastolic function of the left ventricle[141,142]. Notably, ACE2 also has immunomodulatory properties, both indirectly by decreasing Ang-II concentration, which favors inflammation and interacts with macrophages[56,143].

For the first time, Sama *et al*[140] assessed the levels of ACE2 in circulation in a group of patients with heart failure that included 1485 males and 537 females from Europe. The findings were confirmed in a separate survey. The ACE2 plasma levels were found to be increased in males in both the cohorts explaining high susceptibility and fatality rate in males [140], and this elevated ACE2 concentration was consistent with a higher prevalence and severity of COVID-19[2,3,140]. Consequently, elevated ACE2 expression may heighten the sensitivity to COVID-19 and may intensify the infection by augmenting the viral load within the cell. Because the administration of ARBs/ACEi upregulates the expression of ACE2, there are concerns regarding the usage of these drugs, which have not yet been verified[40,144-146].

Secondly, SAR-CoV-2 infection downregulates ACE2 and thus increases the Ang-II levels, which stimulates AT1R. Acute respiratory distress syndrome (ARDS), heart and/or lung damage, and other COVID-19 problems may be primarily brought on by elevated Ang-II activity[145,147,148]. This demonstrates the protective function of ACE2, suggesting that the use of ARBs may mitigate the organ damage caused by COVID-19. When the ACE2 concentration in two heart failure cohorts (validation cohort: 1123 males and 575 females; index cohort: 1485 males and 537 females) was assessed, there was no correlation seen between RAAS inhibitor medication and increased plasma ACE2 concentration [140]. This study suggested that ARB/ACEi treatment may not increase COVID-19 vulnerability through elevated plasma ACE2 levels[140].

At present several things remain uncertain regarding cardiac injury before COVID-19. The clinical implication of detecting myocardial injury remains uncertain as cardiac injury can only be diagnosed using biomarker measurements. At present there is no specific treatment. Regarding the use of ARBs and/or ACEi, no evidence is found to support a higher risk of COVID-19 or severity in individuals who are on these medications. Therefore, their use should be continued until any study proves its harmful effects.

Possible mechanisms involved in myocardial injury during COVID-19

There are several mechanisms through which COVID-19 may promote myocardial damage. COVID-19 has harmful effects caused by sympathetic activation, fever, and tachycardia with elevated energy expenditure and myocardial oxygen consumption[149]. Prolonged bed rest is another consequence of acute COVID-19, and this infection can cause thrombosis, a serious complication[150]. Another feature of COVID-19 is hypoxemia, which is associated with elevated oxidative stress brought on by the production of reactive oxygen species, damage to the mitochondria, intracellular acidosis, and even cell apoptosis[11,88,149,151].

Several more pathways are connected to the aberrant inflammatory reactions brought on by COVID-19. After 7-10 d from the start of COVID-19, a hyperinflammatory reaction with cytokine release (cytokine storm) may happen. These reactions may result in cardiac failure, thromboembolic events, shock, renal failure, and possibly multiorgan failure. They may increase the risk of pneumonia and ARDS[5,7,64,129]. The oxidative stress and activation of the inflammatory response may lead to severe clinical conditions in these patients, and this might elevate the possibility of mortality in these individuals with heart failure[152,153].

An association was seen among different inflammatory markers (like ferritin, C-reactive protein, IL-6, and d-dimer) and major complications and high mortality[5,7,129]. A constant increase in inflammatory markers was also associated with myocardial damage in line with hyperinflammation, which leads to cardiac dysfunction[64,129]. Currently, the beneficial roles of anti-inflammatory therapies in COVID-19 are being studied[154,155]. On the other hand, drugs working on endothelial functions, like ACEi or ARBs and statins may also be beneficial[156].

DIFFERENT STRATEGIES FOR THE TREATMENT OF COVID-19

COVID-19 treatment strategies of T2DM patients

Treatment plans for patients with diabetes were determined based on their blood glucose and/or glycated hemoglobin levels, as well as their urine ketone levels, at the time of admission. At the time of admission, several factors were considered, including age, nutritional status, food intake, the existence of different organ dysfunctions, and conditions related to the heart and brain. This took into account both the potential risks associated with fluctuations in blood glucose levels and the severity of the patient's condition[157].

As per the guidelines provided by the United Kingdom National Diabetes Inpatient COVID Response Group[158,159], it was recommended that all patients hospitalized with COVID-19 should diligently monitor their blood glucose levels. This would enable prompt identification of any changes in glucose levels. Individuals who had a history of diabetes or who showed up with blood glucose levels more than 12 mmol/L were regularly checked (2-4 times per hour) and treated for their diabetes. The aim for blood glucose management might be changed to 7-12 mmol/L if insulin therapy is implemented and blood glucose levels continue to surpass the 12 mmol/L threshold or if oral medication is not practical. When treatment for COVID-19-related symptoms did not provide the desired outcomes and real blood glucose readings did not match expectations, more frequent blood glucose monitoring (4-6 times per hour) was recommended. When blood glucose levels exceeded 15 mmol/L, subcutaneous administration of short-acting insulin was performed as required.

Dexamethasone emerged as a prominent treatment option for severe COVID-19 cases due to its demonstrated ability to mitigate fatalities among individuals requiring ventilator and oxygen therapies. However, given its classification as a glucocorticoid, special caution was urged for COVID-19 patients also managing diabetes when utilizing dexamethasone, to avert the onset of ketoacidosis. In scenarios involving dexamethasone treatment, patients and healthcare practitioners were advised to increase the frequency of blood glucose level assessments to ensure optimal glycemic control. Alternatively, consideration for alternate therapeutic approaches was recommended for patients grappling with uncontrolled hyperglycemia[160].

COVID-19 treatment strategies involving ACE2/RAAS system: Recombinant human ACE2 treatment therapy

Adenoviral ACE2, also known as recombinant human ACE2 (rhACE2), has been used as a therapeutic intervention in animal models of sickness and a human trial including 44 patients with ARDS[161]. rhACE2 is a crucial negative regulator of Ang-II and inhibits harmful cardiac remodeling[142]. When rhACE2 significantly increases ACE2 activity in the circulation, Ang-II levels significantly decrease and Ang-(1-7) synthesis from Ang-II increases. A chimeric fusion of immunoglobulin fragment Fc region restored induced hypertension in mice and increased total Ang-II-conversion activities in blood by up to 100 times.

For COVID-19 patients, Verma *et al*[162] suggested a combination treatment that included rhACE2 and GapmeR technology. While rhACE2 stops the virus from infecting host cells, GapmeR is an antisense single-stranded DNA molecule that binds to the SARS-CoV-2 RNA. The resulting DNA-RNA hybrid is then destroyed by intracellular RNAase H[162]. For the treatment of this sickness, cyclodextrin soluble ACE2 (sACE2) insertion has been suggested as an alternative method[163]. Many aerosolized sACE2 compounds that may be inhaled directly into the lungs, intravenous sACE2 infusions, and ocular and nasal drops made from cyclodextrin sACE2 inclusion compounds have all been proposed as treatments for COVID-19[164].

Use of RAAS inhibitors

Recent trials have shown that RAAS inhibitors and increased ACE2 levels are beneficial for COVID-19 individuals. As mentioned earlier, ACE2 is downregulated when it binds to the SARS-CoV-2 S-protein, which raises Ang-II levels. Breathing problems and lung congestion result from this[148,165]. Therefore, some drugs that increase ACE2 concentration may help lessen the severity of COVID-19[106]. Patients treated with RAS inhibitor had a lower mortality rate than patients taking other hypertensive drugs, according to many retrospective studies on people with COVID-19 and hypertension[166,167].

Lam *et al*[168] showed that hypertensive COVID-19 patients who continued to use ACEi or ARBs had better clinical results and were advised to stick with their current course of treatment. Meng *et al*[169] separated their study subjects into two separate groups: Those taking RAS inhibitors (including 17 participants); and those not taking RAS inhibitors (including 25 participants). As opposed to those receiving on-RAS inhibitors, they found that those receiving RAS inhibitors had lower viral loads, lowered serum IL-6, increased blood $CD8^+$ and $CD3^+$ T cells, and decreased sickness severity. Thus, medications such as RAS inhibitors have been associated with better clinical results in COVID-19 hypertension patients[14].

COVID-19 treatment strategies of T2DM patients involving the ACE2/RAAS system

Several clinical trials have been performed for the efficacy and safety of potential alternatives, including arbidol, chloroquine phosphate, tocilizumab, ribavirin, and remdesivir, among others. Chloroquine and its hydroxy analog hydroxychloroquine are a promising pharmacological option for patients with diabetes as it is a broad-spectrum antiviral drug and is commonly used to treat malaria and autoimmune diseases. Chloroquine increases endosomal pH and inhibits ACE2 glycosylation, thereby blocking the SARS-CoV entry in the host cell[170]. It has been shown that chloroquine increases the C-peptide response, which indicates enhanced function of the pancreatic β cell[171]. Several studies showed improved glycemic control by hydroxychloroquine in decompensated diabetic patients, which were refractory to other antidiabetic drugs[171,172].

The severity of the condition, age, the existence of comorbidities, and problems associated with diabetes, among other things, should all be taken into consideration while developing therapeutic methods and ideal glucose levels. A greater number of COVID-19 tests in outpatient diabetes clinics may exhibit a positive impact on their outcome.

Identifying the epigenetic signatures linked to COVID-19 and their changes during viral entry and infection (such as going from asymptomatic to mildly symptomatic, severe infection, and long-lasting symptoms) could be helpful in facilitating prompt diagnosis and the advancement of treatments that could lessen the severity of the virus and its associated mortality. Major metabolic issues such as T2DM, hypertension, and CVD are factors that lead to COVID-19 patient death. Finding epigenetic markers linked to these comorbidities and their impact on COVID-19 severity may also be helpful in guiding treatment to prevent the development of sequelae that increase the catastrophic death rate associated with COVID-19[173].

CONCLUSION

Early diagnosis, early isolation, and early management might prove to be important in controlling COVID-19. ACE2 is the major receptor for the SARS virus and the interaction between the S-protein and ACE2 is proposed to be the potential factor for infectivity[174,175]. There are concerns regarding the use of RAAS blockers as they may alter ACE2 and whether the altered ACE2 expression is responsible for COVID-19 virulence[39,40,124,146]. There are uncertainties regarding the association of elevated ACE2 expression and infectivity of SARS-CoV-2. The available evidence suggests that RAAS inhibitors should be continued until it is demonstrated that there is an association of ARB/ACEi use with increased severity of COVID-19.

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FOOTNOTES

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MINIREVIEWS

Are treatment options used for adult-onset type 2 diabetes mellitus (equally) available and effective for children and adolescents?

Nevena Krnic, Vibor Sesa, Anna Mrzljak, Maja Cigrovski Berkovic

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Abstract

Youth-onset type 2 diabetes mellitus (T2DM), influenced by an increase in obesity, is a rising problem worldwide. Pathophysiological mechanisms of this early-onset T2DM include both peripheral and hepatic insulin resistance, along with increased hepatic fasting glucose production accompanied by inadequate first and second-phase insulin secretion. Moreover, the incretin effect is reduced. The initial presentation of type 2 diabetes can be dramatic and symptoms may overlap with those of type 1 diabetes mellitus. Therefore, immediate therapy should address hyperglycemia and associated metabolic derangements irrespective of ultimate diabetes type, while further therapy adjustments are prone to patients' phenotype. New agents with proven glycemic and beyond glycemia benefits, such as Glucagon-like polypeptide 1 receptor agonists and Sodium-glucose cotransporter-2 inhibitors, used in the adult population of T2DM patients, might become increasingly important in the treatment armamentarium. Moreover, metabolic surgery is an option for markedly obese (body mass index > 35 kg/m^2) children and adolescents suffering from T2DM who have uncontrolled glycemia and/or serious comorbidities when lifestyle and pharmacologic interventions fail. In this mini-review, we will discuss the potential of treatment options considering new data available from randomized control trials, including individuals with adult-onset type diabetes mellitus.

Key Words: Youth-onset type 2 diabetes mellitus; Treatment; Complications; Glucose lowering agents; Extra-glycemic benefit

Core Tip: Youth-onset type 2 diabetes mellitus (T2DM) is a growing health problem. The incidence of youth-onset T2DM is especially high in overweight/obese individuals, in some ethnic groups with higher preponderance in female sex. Due to more aggressive course of T2DM in youth and earlier development of chronic complications, the stricter metabolic control targeting both glycemia and other vascular risk factors might be necessary. Newer agents with cardiovascular benefits and weight losing potential might become increasingly important in treatment of youth-onset T2DM. Moreover, future studies should focus on different approaches according to gender and use of new technologies in glucose monitoring.

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INTRODUCTION

Youth-onset type 2 diabetes mellitus (T2DM) is increasing in incidence and prevalence. There is a strong predilection for its development in certain ethnic groups[1] and evermore so, the development of T2DM is related to the cumulative effect of early onset persistent obesity[2]. Despite male predominance among adolescents with prediabetes[3], there is a higher prevalence of T2DM in females[4]. Due to specific and unfavorable metabolic profiles encompassing insulin resistance and early β-cell decline, youth-onset T2DM is often accompanied by chronic end-stage complications, which become apparent much earlier in life[4,5]. Especially important are cardiovascular complications, which are not only a consequence of early-onset hyperglycemia but also of accompanying comorbidities, making treatment decisions difficult [6]. Currently, only a few therapeutic agents gained approval from the regulatory agencies for the treatment of youth-onset T2DM. Decision on their use and combinations often depends on the presenting clinical features, hemoglobin A1c (HbA1c) and hyperglycemia level, and the presence of catabolism (diabetic ketoacidosis or hyperosmolar hyperglycemic state). Moreover, it also depends on the long-term efficacy and, in the newer time, due to the availability of agents with cardiovascular and renal benefits, on the patient's risk stratification and presence of comorbidities. Although there is a female gender predilection for T2DM development and a tendency for resolution of dysglycemia in male patients following puberty[7], there are no recommendations for different treatment approaches in male and female patients. Most treatment practice for youth-onset T2DM is derived from adult patients. However, they do not necessarily have the same pathophysiological features and therapeutic effects might not be the same as in adults. The aim of treatment in youth with T2DM is agreed at HbA1c < 7.0%[8], but several studies demonstrated a more aggressive course of disease in younger patients, suggesting stricter metabolic control might be necessary[9]. All available treatment modalities have comparable glucose-lowering effects[8] of HbA1c reduction by 1%-2%, but no data regarding favorable effects on other metabolic complications of T2DM and obesity. Furthermore, pharmacological and nonpharmacological therapy failure is more common in adolescents (first-line treatment with metformin and intensive lifestyle changes are suboptimal in more than 50% of youth within 2 years of diagnosis), necessitating the need for treatment intensification[10,11].

Metformin

Metformin remains the pharmacological cornerstone of T2DM treatment in children and adolescents with T2DM, according to international guidelines[8,12]. It is advised as a first-line treatment in case of newly diagnosed T2DM in children and youth who present without ketoacidosis or hyperosmolar hyperglycemic state and if HbA1c < 8.5%. In contrast, it can be combined with basal insulin as a starting therapy when HbA1c exceeds 8.5%, with no signs of hypoinsulinemia[13].

Improvement in HbA1c ranges from 1 to 2%, but metabolic effects also include improved insulin sensitivity and potential beneficial effects on cardiovascular function, while the effects on reduction of body weight and waist circumference are limited[14]. In adults, metformin increases insulin sensitivity and delays β-cell decline. Compared to adults, metformin therapy does not favor residual β-cell function in children and adolescents[15], presumably due to more apparent hepatic insulin resistance earlier in the disease course[16]. Therefore, there is no current data to support the use of metformin in adolescents with prediabetes for the prevention of T2DM development[17]. Moreover, expectations of its long-term effects on glucose lowering in youth-onset T2DM alone or in combination with lifestyle interventions or insulin are modest, while its modulation of the entero-insular axis *via* gut microbiome is of a neglectable size. In addition, up to date, there is no evidence of its effect on reducing visceral or hepatic fat or lipolysis in children and adolescents with T2DM[11] (Figure 1).

Thiazolidinediones

Rosiglitazone was evaluated in youth with T2DM as an add-on therapy to metformin[18], with superior improvement in insulin sensitivity and fewer adverse effects[19] as compared to metformin alone, but so far, it has not gained approval for T2DM treatment in children and adolescents. In addition, rosiglitazone has been strongly restricted or even

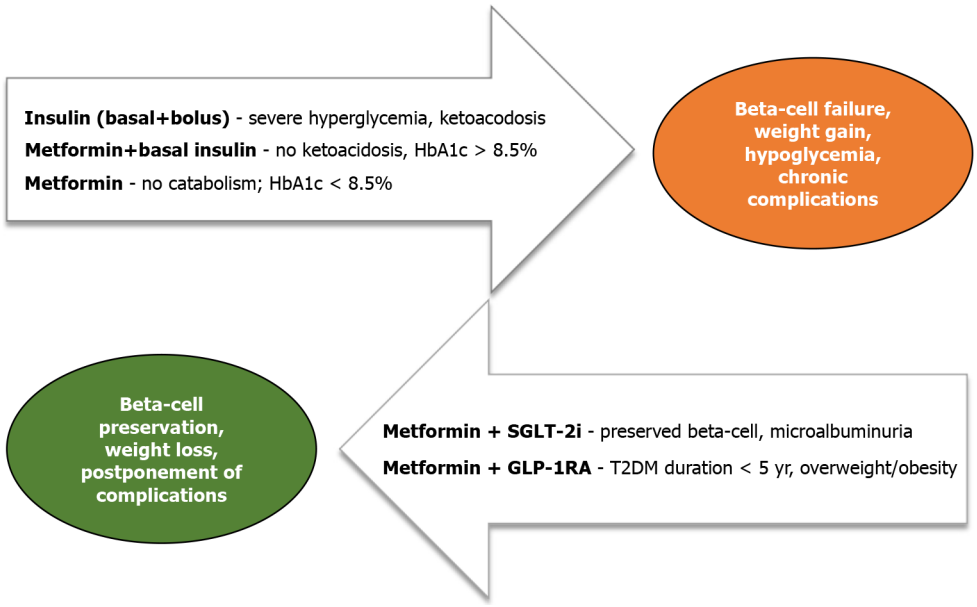

Figure 1 Paradigm shift in youth-onset type 2 diabetes mellitus treatment. T2DM: Type 2 diabetes mellitus; HbA1c: Hemoglobin A1c; SGLT-2i: Sodium-glucose cotransporter-2 inhibitors; GLP-1RA: Glucagon-like polypeptide 1 receptor agonists.

withdrawn from the market in most countries due to concerns about its cardiovascular safety; therefore, its use in adult T2DM is limited[20]. On the other hand, pioglitazone, yet another thiazolidinedione, has an important role in the treatment of adult T2DM patients with metabolic syndrome and cardiovascular risk, but its use in youth-onset T2DM is scarce and not approved[21].

Insulin

Several therapy modalities using different types of insulin are used in the treatment of youth-onset T2DM. Insulin can be used as initial therapy in severe presentation of T2DM (ketosis/ketoacidosis or HbA1c > 8.5%)[8] or additional therapy if other therapeutic options fail to achieve improvement in metabolic control. However, insulin can lead to weight gain, which can perpetuate obesity and subsequent metabolic complications. The addition of insulin to youth with T2DM already treated with different treatment modalities (metformin alone/metformin + rosiglitazone/metformin + lifestyle interventions) led to variable therapeutic effects (only 33.2% of participants had consistent HbA1c decrease of ≥ 0.5%) [22], in addition to the need of treatment intensification with more complex regimens (basal bolus)[13]. Insulin is advised primarily in the form of multiple daily injections. At the same time, there are no studies done on youth-onset T2DM with insulin delivered *via* pump therapy with hybrid closed-loop technology, although data from adult T2DM are promising [23].

Glucagon-like polypeptide 1 receptor agonists

Glucagon-like polypeptide 1 receptor agonists (GLP-1RA), liraglutide, exenatide and dulaglutide, once daily or once weekly, have recently been approved for the treatment of T2DM in children and adolescents. The therapeutic benefit includes glucose-lowering effect (HbA1c lowering by 0.85% to 1.4% after 24 to 26 wk of therapy), while effects on body mass index and waist circumference are less pronounced than that seen in the adult population with T2DM and potentially require more prolonged duration of treatment (more than 52 wk)[24-26]. Moreover, a rebound is seen after discontinuation of treatment[27-29]. Nonetheless, one might speculate that early introduction of GLP-1RAs, in combination with metformin, might be beneficial for youth-onset T2DM, especially in case of early-stage disease, lasting for less than 5 years, while it would improve β-cell function, in addition to targeting insulin resistance and obesity.

Sodium-glucose cotransporter-2 inhibitors

Sodium-glucose cotransporter-2 inhibitors (SGLT-2i), empagliflozin, has been approved for treating children and adolescents with T2DM since June 2023, alone or in combination with metformin[29]. The reduction in HbA1c of 1.13% was demonstrated in clinical trials, and similar to GLP-1RAs, the weight-lowering effect is minimal compared to the one seen in adults with T2DM[30]. Although no data is available in youth with T2DM, it could be assumed that beneficial effect on cardiovascular function and renal disease, as seen in adults, would also be demonstrated in youth with T2DM [31,32]. SGLT-2i promote urinary glucose excretion with compensatory increases in rates of gluconeogenesis and ketosis. How this transcribes in glucose management of young-onset T2DM is to be seen, while there is a defect in gluconeogenesis regulation. Moreover, the problem of euglycemic ketosis must not be neglected.

Use of technological devices

As compared to the frequent use of different technological devices among children and adolescents with type 1 diabetes, there are no current recommendations for similar use among youth with T2DM. Some studies have proven that

continuous or intermittent glucose monitoring devices (CGM) are beneficial for lifestyle modification interventions[33]. Furthermore, it was demonstrated that greater fasting glucose variability during the first year following the diagnosis of T2DM is highly predictive of deterioration of β-cell function and development of comorbidities in following years[34]. CGM might help to detect those patients and intensify their treatment in the early phase of T2DM. The indications for using devices for CGM with different types of treatment or cost-effectiveness have not yet been established, and health insurance policies do not cover expenses in most countries.

Metabolic surgery

Metabolic surgery is becoming an important tool in treating obesity in youth with type 2 diabetes, although numbers of treated patients are rather small and data on long-term safety and durability of treatment success still lacking[35]. The most commonly used methods Roux-en-Y gastric bypass, and vertical sleeve gastrectomy are highly effective, and weight reduction up to 73% is reported, together with T2DM remission after 3-years of follow-up, which is similar to that observed in adult T2DM population[36]. Timing of metabolic surgery is an important issue, and is still debatable, but many authorities argue that intervention earlier during adolescence, would be more beneficial in terms of offering comprehensive metabolic remission and fewer and/or postponed vascular complications in the future[37]. Finally, meticulous surveillance and strong family support is necessary to prevent nutritional deficiencies and potential regain of weight after bariatric surgery[38,39].

CONCLUSION

Although pharmacotherapy of adult-onset T2DM is diverse and efficient both in glucose management and offering cardiovascular and renovascular benefits, currently, there are only four medication classes for youth-onset T2DM: Historical agents such as metformin, insulin, and newer GLP-1RAs (liraglutide, exenatide and dulaglutide) and SGLT-2i empagliflozin. The poor long-term durability of metformin and insulin with the risk of hypoglycemia, weight gain and no potential for β-cell preservation might lead to a paradigm shift and more robust use of agents beyond glycemic effects. Whether their efficiency and long-term safety will be comparable to that seen in adult T2DM and the preferable timing and best combinations for youth-onset T2DM remain to be seen.

FOOTNOTES

Author contributions: Krnic N was involved in conceptualization of the study; Cigrovski Berkovic M was responsible for data curation; Krnic N and Mrzljak A drafted the original version of the manuscript; Mrzljak A and Sesa V reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Prevalence and risk factors of wound complications after transtibial amputation in patients with diabetic foot

Young Uk Park, Seong Hyuk Eim, Young Wook Seo

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Abstract

BACKGROUND

Diabetic foot (DMF) complications are common and are increasing in incidence. Risk factors related to wound complications are yet to be established after transtibial amputation under the diagnosis of DMF infection.

AIM

To analyze the prognosis and risk factors related to wound complications after transtibial amputation in patients with diabetes.

METHODS

This retrospective cohort study included seventy-two patients with DMF complications who underwent transtibial amputation between April 2014 and March 2023. The groups were categorized based on the occurrence of wound complications, and we compared demographic data between the complication group and the non-complication group to analyze risk factors. Moreover, a multivariate logistic regression analysis was performed to identify risk factors.

RESULTS

The average follow-up period was 36.2 months. Among the 72 cases, 31 (43.1%) had wound complications. Of these, 12 cases (16.7%) received further treatment, such as debridement, soft tissue stump revision, and re-amputation at the proximal level. In a group that required further management due to wound complications after transtibial amputation, the hemoglobin A1c (HbA1c) level was 9.32, while the other group that did not require any treatment had a 7.54 HbA1c level. The prevalence of a history of kidney transplantation with wound complications after transtibial amputation surgery in DMF patients was significantly greater than in cases without wound complications (P = 0.02). Other factors did not show significant differences.

CONCLUSION
Approximately 43.1% of the patients with transtibial amputation surgery experienced wound complications, and 16.7% required additional surgical treatment. High HbA1c levels and kidney transplant history are risk factors for postoperative wound complications.

Key Words: Diabetic foot; Transtibial amputation; Wound complications; Risk factor

Core Tip: In this study, 43.1% of the patients with transtibial amputation surgery experienced wound complications, and 16.7% necessitated additional wound revision procedures, such as debridement. High hemoglobin A1c (HbA1c) levels (HbA1c > 7.2) and kidney transplant history are risk factors for postoperative wound complications.

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INTRODUCTION

The World Health Organization reported that the estimated number of patients with diabetes reached nearly 425 million in 2017. Unsurprisingly, this led to an increasing number of diabetes-related complications[1]. Diabetic foot (DMF) is one of the most devastating, if not the most critical, complications of diabetes mellitus (DM)[2]. DM foot infection complications range from 10% to 25% throughout the lifetime of one DM patient[3]. It is also the main reason for non-traumatic lower extremity amputations[4]. Risk factors for amputation include smoking history, kidney transplantation history, high sugar levels, hyperlipidemia, and ischemia[5,6]. Amputation can be subdivided into minor and major amputations, and transtibial amputations are regarded as major. Post-operative 30-d mortality rates have been reported to be 6%-17%; the 1-year mortality rate after major amputation is 69.7%; and the 5-year mortality rate is 34.7%[7-9]. Thorud *et al*[10] reported the overall 5-year mortality rate to be very high among patients with any amputation, ranging from 53% to 100% and from 52% to 80% for patients with major amputations[10].

Major amputation is considered the final therapeutic option; nevertheless, after major amputation, wound complications may persist, necessitating further surgical interventions. Prognosis and risk factors related to wound complications have yet to be established after transtibial amputation under the diagnosis of DMF complications. The purpose of this study was to analyze the prognosis and risk factors related to wound complications after transtibial amputation in patients with diabetes.

MATERIALS AND METHODS

Study design and patients

This study was approved by the Institutional Review Board of Ajou University School of Medicine, Suwon, South Korea. Seventy-two patients with DMF infection underwent transtibial amputations between April 2014 and March 2023. The medical records and photographs stored in Picture Archiving and Communication System (PACS) were analyzed to ascertain the presence of wound complications and to categorize the types of wound complications, all of which were then meticulously documented. The Size (area, depth), Sepsis, Arteriopathy, and Denervation system was introduced in 1999 and is primarily designed for clinical audits[11]. The system was initially validated in 2004, and to enhance the classification of ulcers for prospective research, certain criteria that were absent in the UT system were subsequently incorporated[12]. In this study, the criteria for necrosis and infection were defined as grade 2 or higher based on the previously published guidelines. The criterion for wound necrosis was defined as wound necrosis over 1 cm^2, and wound infection was defined as suspected local infection, such as pus discharge or cellulitis over 1 cm^2[12,13].

Patient details were obtained by analyzing documented electronic medical records and test results. These details included body mass index (BMI), smoking history, kidney transplantation history, dialysis therapy history, lower extremity endovascular intervention history, previous amputation at the same extremity, and the need for stump revision surgery during follow-up period. Additionally, pre-operative erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and hemoglobin A1c (HbA1c) results were collected.

The occurrence of postoperative wound complications (infection, necrosis, *etc*.) after transtibial amputation surgery was classified into two groups and the contribution of each risk factor was analyzed. Group 1 was defined as cases without wound complications after transtibial amputation surgery in DMF patients, and Group 2 was defined as cases with wound complications.

Data analysis

All statistical analyses were performed using SPSS for Windows, version 22.0 (IBM Corporation, Armonk, NY, United States). Univariate and multivariate logistic regression analyses were performed to identify risk factors. Statistical significance was set at $P < 0.05$. The optimal HbA1c cutoff for postoperative wound complications was calculated using the receiver operating characteristic (ROC) curve.

A significant sample size was calculated by the Muller and Buttner method, and Walter *et al*[14] G*Power version 3.1.9.7 software (Franz Faul, Universität Kiel, Germany) was utilized for the calculation, and the sample size was analyzed by setting the odds ratio (OR) to 20.47, R2 other X to 0.25 (moderate association), alpha error probability to 0.05, and power to 0.80[15,16].

RESULTS

Demographic

Seventy-two patients with DMF complications underwent transtibial amputation between April 2014 and March 2023. Of the 72 patients, 48 (66.7%) were male patients and 24 (33.3%) were female patients. The average age was 64.1 years (39-87), the average BMI was 22.6 (14.3-34.3), and the average period of DM was 18.9 years. Right-side surgery was performed in 30 cases (41.7%), left-side surgery was performed in 39 cases (54.2%), and bilateral surgery was performed in 3 cases (4.2%). Regarding renal function, 43 cases (59.7%) did not undergo dialysis, 19 cases (26.4%) underwent dialysis, and 11 cases (15.3%) received kidney transplants. A total of 19 patients smoked (26.4%). Concerning the history of DMF amputation before this transtibial amputation surgery, there were 19 cases (26.4%) wherein minor amputation (below hindfoot level) was done on the same side, the ankle and hindfoot level amputation cases were 4 (5.6%), the opposite transtibial amputation cases were 6 (8.3%), and the opposite minor amputation cases were 6 (8.3%) (Table 1).

Prognosis

The average follow-up period was 36.2 months (confidence interval: 8-72 months). Among the 72 cases, 12 cases (16.7%) were performed with additional wound management (stump revision = 11 cases; transfemoral amputation = 1 case). In 12 cases, wound healing did not progress satisfactorily with a simple dressing alone, necessitating daily debridement to address infection or necrotic tissue. Some cases had to be followed up with delayed suturing after improvement. Among them, one case exhibited infection and soft tissue necrosis extending up to the knee joint, leading to transfemoral amputation. The remaining 11 cases were discharged after achieving wound stabilization and receiving outpatient follow-up observations.

Risk factor

Compared with HbA1c level of Group 1 (7.54), the HbA1c level of Group 2 (9.32) was significantly higher ($P = 0.01$). The optimal HbA1c cutoff for postoperative wound complications was calculated using the ROC curve, and the result was an HbA1c of 7.2 (Figure 1). In cases with HbA1c levels greater than or equal to 7.2, the probability of postoperative wound complications was 31.28 times higher than in those with lower levels ($P < 0.01$). The prevalence of a history of kidney transplantation in Group 2 was significantly greater than that in Group 1 ($P = 0.02$) (OR: 26.22) (Table 2).

In Group 2, the HbA1c level was significantly higher at 8.77 than the HbA1c level of 7.07 in Group 1 ($P = 0.01$) (OR: 29.65). The prevalence of a history of kidney transplantation in Group 2 (33.3%) was significantly higher compared to Group 1 (11.7%) ($P = 0.03$) (OR: 21.24).

No statistically significant difference was observed in the ratio of dialysis in the group comparison related to additional surgery or treatment. Other factors, including CRP, culture results, DM morbidity period, smoking history, and previous amputation history, did not display significant differences.

DISCUSSION

The most important finding of this study is that postoperative wound complications after transtibial amputation are relatively common, thus requiring close observation. Several of these complications lead to the necessity of wound revisions, emphasizing the significance for physicians to acknowledge this aspect and engage in proactive discussions regarding the potential course of the condition with patients.

In this study, 30-d mortality after transtibial amputation was 5 out of 72 cases (6.9%), and 3-year mortality after transtibial amputation was 14 out of 40 cases (37.5%). Previous studies have also reported the survival rates after transtibial amputation surgery. The range of mortality after transtibial amputation ranged from 40% to 82%, and transtibial amputation ranged from 40% to 90%. The 30-d mortality after major amputations appeared to range from about 5.5% to 13.3%[17-19]. Overall, the 5-year mortality rate was very high among patients with any amputation, ranging from 53 to 100% and from 52% to 80% for patients with major amputations[10]. Increased 5-year mortality was related to old age and kidney function[10,19].

However, there is a lack of reported studies on wound prognosis after transtibial amputation surgery. Among the 72 cases, 31 (43.1%) had wound complications (infection = 8 cases, necrosis = 19 cases, infection and necrosis = 4 cases) in this study. Among the 72 cases, 12 cases (16.7%) were performed with additional wound management. There were 11 cases of stump revision and 1 case of additional surgery with transfemoral amputation (above-knee amputation). The

Table 1 Demographics, *n* (%)

		Group 1 (*n* = 41)	Group 2 (*n* = 31)	*P* value
Age (years), mean ± SD				0.66
		66.0 (9.9)	61.4 (10.9)	
	Median (Q1 to Q3)	65.0 (59.0 to 74.0)	61 (56.0 to 66.5)	
Sex				0.93
	Female	13 (31.7)	11 (35.5)	
	Male	28 (68.3)	20 (64.5)	
BMI, mean ± SD				0.64
		22.4 (3.8)	22.8 (3.8)	
	Median (Q1 to Q3)	22.5 (19.5 to 24.4)	22.2 (19.5 to 25.7)	
Location				0.92
	Right	16 (39.0)	14 (45.2)	
	Left	23 (56.1)	16 (51.6)	
	Both	2 (4.9)	1 (3.2)	
Dialysis				0.86
	No	31 (75.6)	22 (71.0)	
	Yes	10 (24.4)	9 (29.0)	
Kidney transplant history				0.02
	No	40 (97.6)	21 (67.7)	
	Yes	1 (2.4)	10 (32.3)	
Smoking				0.71
	No	29 (70.7)	24 (77.4)	
	Yes	12 (29.3)	7 (22.6)	
Amputation history				0.84
	No	22 (53.7)	15 (48.4)	
	Yes	19 (46.3)	16 (51.6)	
HTN				0.06
	No	17 (41.5)	4 (12.9)	
	Yes	24 (58.5)	27 (87.1)	
Endovascular intervention history				0.38
	No	29 (70.7)	18 (58.1)	
	Yes	12 (29.3)	13 (41.9)	
HbA1		7.54	9.32	0.01
HbA1c ≥ 7.2				0.01
	No	18 (43.9)	4 (12.9)	
	Yes	23 (56.1)	27 (87.1)	

BMI: Body mass index; HTN: Hypertension; HbA1c: Hemoglobin A1c.

reasons for wound revision were infection in 4 cases (33.3%), necrosis in 1 case (8.4%), and infection with necrosis occurring concomitantly in 7 cases (58.3%). Since wound complications requiring wound revision may occur in 16.7% of patients after transtibial amputation surgery, it is important to fully explain the progress to the patient before transtibial amputation surgery. In addition, proper wound management after surgery is important, which is thought to increase the length of hospital stay and the subsequent occurrence of other complications such as pneumonia. However, additional major amputation was performed in 1 case out of 72 (1.3%), so wound recovery can be expected through appropriate

Table 2 Risk factors

		Univariable		Multivariable_all	
		OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age (years)		0.96 (0.91-1.00)	0.066	1.01 (0.93-1.09)	0.866
Sex					
	Female	-		-	
	Male	0.84 (0.31-2.29)	0.737	0.53 (0.08-2.92)	0.476
BMI		1.03 (0.91-1.17)	0.630	1.10 (0.87-1.45)	0.439
Location					
	Right	-		-	
	Left	0.80 (0.30-2.08)	0.640	0.62 (0.14-2.65)	0.525
	Both	0.57 (0.02-6.60)	0.662	0.11 (0.00-3.08)	0.239
Kidney transplant history					
	No	-		-	
	Yes	19.05 (3.32-361.72)	0.006[a]	26.22 (2.36-811.46)	0.020
Smoking					
	No	-		-	
	Yes	0.70 (0.23-2.04)	0.525	0.26 (0.04-1.52)	0.150
ICU					
	No	-		-	
	Yes	0.64 (0.23-1.71)	0.379	0.20 (0.03-1.09)	0.085
HTN					
	No	-		-	
	Yes	4.78 (1.52-18.42)	0.012	6.19 (1.20-44.22)	0.054
Endovascular intervention history					
	No	-		-	
	Yes	1.75 (0.66-4.72)	0.266	2.86 (0.63-16.12)	0.193
HbA1c ≥ 7.2					
	No	-		-	
	Yes	5.28 (1.69-20.33)	0.007[a]	31.28 (5.04-355.9)	0.001

[a]$P < 0.05$.

BMI: Body mass index; ICU: Intensive care unit; HTN: Hypertension; HbA1c: Hemoglobin A1c; OR: Odds ratio; CI: Confidence interval.

wound management. Various risk factors related to DMF ulcers and DMF amputations are known[1,20-25]. Cervantes-García and Salazar-Schettino[20] found that males and smoking history, which were indicated as risk factors for amputation in diabetes-related foot ulceration, were also identified as risk factors for amputation in DMF infection in a meta-analysis[20]. Diabetic complications, including peripheral Arterial Disease, peripheral neuropathy, nephropathy, and severe infection, were identified as major causes of amputation[1,20,21]. However, Sen *et al*[23] reported that DM neuropathy was not associated with DMF amputation risk factors. Although nephropathy is important in the development of DMF ulcers, this complication was not found in this meta-analysis to be the cause of amputation in patients with DMF infection (DFI)[22,23]. Based on studies conducted by Aziz *et al*[24] and Shojaiefard *et al*[25], elevated average leukocytosis, CRP, ESR, HbA1c levels, and hyperglycemia have been identified as risk factors for amputation. Park *et al*[26] reported that high glucose levels (> 300 mg/dL) and hypotension at admission are identified as independent risk factors for limb loss in patients with necrotizing fasciitis.

Furthermore, there is limited understanding of the risk factors associated with wound complications following transtibial amputation surgery. Various risk factors were analyzed in this study. In this study, high HbA1c and kidney transplantation history were analyzed as risk factors for postoperative wound complications after transtibial amputation surgery. Sinacore reported that wound healing is delayed due to the use of immunosuppressive agents after trans-

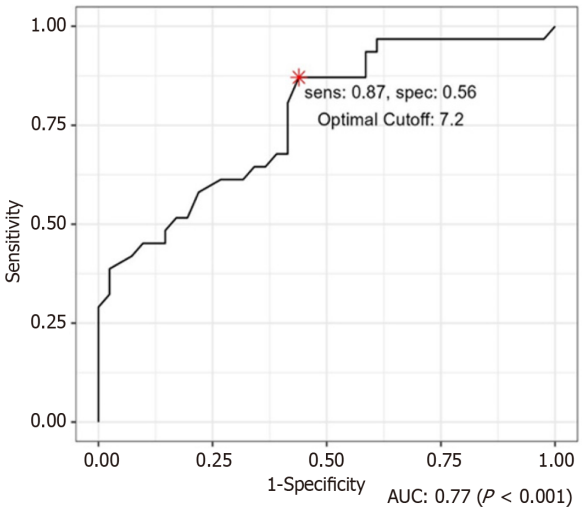

Figure 1 Receiver operating characteristic curve for optimal cutoff of hemoglobin A1c. AUC: Area under the curve.

plantation[27]. Seo *et al*[28] reported that post-pancreas transplantation, 6.9% of individuals developed DMF ulcers, and 3.2% developed Charcot arthropathy. A study by Sharma *et al*[29], involving 235 kidney transplant patients, revealed a 15% incidence of DMF ulcers. In their multivariate analysis, Uçkay *et al*[30] observed that the presence of chronic, enhanced immune suppression, compared to its absence, is linked with an increased likelihood of clinical failures in DFI cases, indicated by a hazard ratio of 1.5 and a 95% confidence interval ranging from 1.1 to 2.0. This suggests that a consistently elevated level of immune suppression may act as an independent factor increasing the risk of unsuccessful treatment outcomes in DFI[30]. According to Huang *et al*[31], the wound healing process in DMF patients as intricate, involving factors such as elevated blood sugar levels, reduced blood flow, low oxygen levels, heightened inflammatory response, and ongoing infections. Based on these prior studies, this study also identifies risk factors for wound complications associated with impaired wound healing, such as immunosuppressive agent usage following kidney transplantation. No significant differences ($P > 0.05$) were found in age, gender, duration of diabetes, BMI, smoking status, whether the patient received treatment in the intensive care unit, or whether they underwent dialysis as risk factors for surgical wound complications.

The present study had limitations. There is no research conducted on the correlation between risk factor adjustment and a reduction in the occurrence of complications. Further research is required to investigate this matter. This study is retrospective in design and carries inherent limitations when compared to prospective studies. Nevertheless, within this study, clinical photographs were serially captured for all patients before and after surgery, as well as during each wound management, and these images were stored in the PACS for documentation. This approach facilitated a precise evaluation of complication presence and wound status, utilizing not only medical records but also PACS clinical photos. Due to the analysis of actual wound status using PACS, which could be missed in medical records, this study concludes that the high incidence of wound complications after transtibial amputation surgery is the reason for its observation.

CONCLUSION

In this study, 43.1% of the patients with transtibial amputation surgery experienced wound complications, and 16.7% necessitated additional wound revision procedures, such as debridement. High HbA1c levels (HbA1c > 7.2) and kidney transplant history are risk factors for postoperative wound complications.

ARTICLE HIGHLIGHTS

Research background

Diabetic foot (DMF) complications are common and are increasing in incidence. Risk factors related to wound complications are yet to be established after transtibial amputation under the diagnosis of DMF infection.

Research motivation

The purpose of this study was to analyze the prognosis and risk factors related to wound complications after transtibial amputation in patients with diabetes.

Research objectives

Having knowledge of the research findings on the prevalence and risk factors of wound complications after transtibial

amputation in patients with DMF, we can utilize this information in a clinical setting for purposes such as predicting patient outcomes and providing explanations to patients.

Research methods

Seventy-two patients with DMF infection underwent transtibial amputations between April 2014 and March 2023. The medical records and photographs stored in Picture Archiving and Communication System were analyzed to ascertain the presence of wound complications and to categorize the types of wound complications. The occurrence of postoperative wound complications after transtibial amputation surgery was classified into two groups and the contribution of each risk factor was analyzed. Group 1 was defined as cases without wound complications after transtibial amputation surgery in DMF patients, and Group 2 was defined as cases with wound complications.

Research results

Among the 72 cases, 12 cases (16.7%) were performed with additional wound management (stump revision = 11 cases; transfemoral amputation = 1 case). In 12 cases, wound healing did not progress satisfactorily with a simple dressing alone, necessitating daily debridement to address infection or necrotic tissue. Compared with hemoglobin A1c (HbA1c) level of Group 1 (7.54), the HbA1c level of Group 2 (9.32) was significantly higher (P = 0.01). The optimal HbA1c cutoff for postoperative wound complications was calculated using the receiver operating characteristic curve, and the result was an HbA1c of 7.2. The prevalence of a history of kidney transplantation in Group 2 was significantly greater than that in Group 1 (P = 0.02) In Group 2, the HbA1c level was significantly higher at 8.77 than the HbA1c level of 7.07 in Group 1 (P = 0.01) [odds ratio (OR): 29.65]. The prevalence of a history of kidney transplantation in Group 2 (33.3%) was significantly higher compared to Group 1 (11.7%) (P = 0.03) (OR: 21.24).

Research conclusions

In this study, 43.1% of the patients with transtibial amputation surgery experienced wound complications, and 16.7% necessitated additional wound revision procedures. High HbA1c levels (HbA1c > 7.2) and kidney transplant history are risk factors for postoperative wound complications.

Research perspectives

No research has been conducted yet on the correlation between adjusting risk factors and reducing complications, highlighting the need for future studies in this area.

FOOTNOTES

Author contributions: Park YU and Seo YW designed the research study, and performed the research; Park YU, Seo YW and Eim SH analyzed the data and wrote the manuscript; All authors have read and approve the final manuscript.

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Informed consent statement: The study is retrospective and does not impact human subjects, thereby not requiring consent forms.

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Abstract

BACKGROUND

Age is a significant risk factor of diabetes mellitus (DM). With the develop of population aging, the incidence of DM remains increasing. Understanding the epidemiology of DM among elderly individuals in a certain area contributes to the DM interventions for the local elderly individuals with high risk of DM.

AIM

To explore the prevalence of DM among elderly individuals in the Lugu community and analyze the related risk factors to provide a valid scientific basis for the health management of elderly individuals.

METHODS

A total of 4816 elderly people who came to the community for physical examination were retrospectively analyzed. The prevalence of DM among the elderly was calculated. The individuals were divided into a DM group and a non-DM group according to the diagnosis of DM to compare the differences in diastolic blood pressure (DBP) and systolic blood pressure (SBP), fasting blood glucose, body mass index (BMI), waist-to-hip ratio (WHR) and incidence of hypertension (HT), coronary heart disease (CHD), and chronic kidney disease (CKD).

RESULTS

DM was diagnosed in 32.70% of the 4816 elderly people. The BMI of the DM group (25.16 ± 3.35) was greater than that of the non-DM group (24.61 ± 3.78). The WHR was 0.90 ± 0.04 in the non-DM group and 0.90 ± 0.03 in the DM group, with no significant difference. The left SBP and SBP in the DM group were 137.9 mmHg ± 11.92 mmHg and 69.95 mmHg ± 7.75 mmHg, respectively, while they were 126.6 mmHg ± 12.44 mmHg and 71.15 mmHg ± 12.55 mmHg, respectively, in the non-DM group. These findings indicate higher SBP and lower DBP in DM patients than in those without DM. In the DM group, 1274 patients were dia-

gnosed with HT, accounting for 80.89%. Among the 3241 non-DM patients, 1743 (53.78%) were hypertensive and 1498 (46.22%) were nonhypertensive. The DM group had more cases of HT than did the non-DM group. There were more patients with CHD or CKD in the DM group than in the non-DM group. There were more patients who drank alcohol more frequently (≥ 3 times) in the DM group than in the non-DM group.

CONCLUSION

Older adults in the Lugu community are at a greater risk of DM. In elderly individuals, DM is closely related to high BMI and HT, CHD, and CKD. Physical examinations should be actively carried out for elderly people to determine their BMI, SBP, DBP, and other signs, and sufficient attention should be given to abnormalities in the above signs before further diagnosis.

Key Words: Diabetes mellitus; Type 2 diabetes mellitus; Elderly; Risk factors

Core Tip: Age is a significant risk factor of diabetes mellitus (DM). An investigation focusing on the prevalence and risk factors of DM among elderly individuals is necessary. A total of 4816 elderly people who came to the community for physical examination were retrospectively analyzed in this study. older adults in the Lugu community are at high risk of DM, a disease highly correlated with high body mass index (BMI) and hypertension, coronary heart disease and chronic kidney disease in the elderly population. Therefore, physical examinations should be actively carried out for elderly individuals in the Lugu community to determine their demographic indices, such as BMI, systolic pressure, and diastolic pressure. Moreover, adequate attention should be given to those with abnormal signs, and blood glucose levels should be further determined.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a multifactorial chronic metabolic disorder characterized by hyperglycemia and insulin resistance[1]. The pathogenesis of this type of DM mainly involves: (1) The lack of insulin secreted by pancreatic cells *in vivo*; and (2) the inability of insulin-sensitive tissues to respond to insulin[2]. In recent years, the number of people with DM has shown a significant upward trend, especially in Asia[3]. China has one of the highest prevalence rates of DM in Asia, and it faces an enormous burden of DM[4], with approximately 11% of the population affected by the disease [5]. In addition to causing problems such as metabolic disorders caused by their own diseases, DM may also increase the risk of patients suffering from retinopathy[6], stroke[7], nephropathy[8], cardiovascular diseases[9] and other diseases.

The aging population and changing lifestyles have made DM more common among older adults[10]. Currently, elderly individuals, especially those aged 60-79 years, have a high incidence of DM, with nearly 50% of patients being aged ≥ 65 years[11]. DM is associated with a high risk of metabolic disorders in elderly individuals, which may increase mortality and reduce quality of life in older adults[12]. It is argued that DM increases the risk of hypoglycemia in older adults, which can lead to cognitive impairment, cardiovascular disease and even death[13]. Therefore, it is necessary to consider the glycemic indices of the elderly population, focusing on elderly individuals who may have DM or have been diagnosed. Understanding the prevalence of DM among elderly people in a certain area and discussing the risk factors related to DM are conducive to carrying out targeted interventions for the local elderly population and developing effective blood glucose (BG) management strategies[14].

The purpose of this study was to explore the prevalence of DM among elderly individuals in the Lugu community and analyze the related risk factors. A total of 4816 older adults who came to the community for physical examination were included, and clinical signs such as body mass index (BMI), waist-to-hip ratio (WHR), and systolic blood pressure (SBP) and diastolic blood pressure (DBP) were collected to diagnose hypertension (HT) and DM. The findings of this study will help to understand the DM status of elderly individuals in the Lugu community and provide corresponding health management suggestions for elderly individuals who come to the community for physical examination.

MATERIALS AND METHODS

Case data

In this study, physical examination data were collected from 37562 people who had physical examinations in the Lugu community, among whom 4816 elderly people (aged ≥ 60 years) were analyzed retrospectively. The weight, height, waist circumference, hip circumference, DBP, SBP, fasting BG (FBG), BMI, and WHR of the elderly individuals were analyzed, as was the incidence of DM and its risk factors.

Detection methods and observation indicators

BP testing: The subjects had no strenuous exercise within 30 min before BP testing. Left SBP and DBP were measured using a noninvasive automatic sphygmomanometer. Subjects who showed SBP was at least 140 mmHg and/or their DBP was at least 90 mmHg at each of the three non-same-day measurements was considered to be HT.

FBG detection: Venous blood was collected at 7:00-9:00 am in the morning after overnight fasting for 8-12 h. The blood sample was placed in a centrifuge tube containing heparin sodium (heparin sodium: blood = 1:9) and centrifuged at 3000 rpm for 10 min, after which the supernatant was carefully aspirated to obtain plasma samples. BG levels were measured by a BS-300 automatic biochemical analyzer. Patients with a FBG ≥ 7.0 mmol/L were further tested to determine whether they had DM.

BMI: The body weight (kg) and height (m) of the subjects were measured, and the BMI (BMI = weight/height^2) was calculated.

WHR: Waist circumference (cm) and hip circumference (cm) were measured to calculate the WHR (WHR= waist circumference/hip circumference).

Data visualization and statistical analysis

The data visualization process of this study was carried out with GraphPad 9.0, and the research data are displayed as pie charts and histograms. This study employed SPSS 22.0 software for statistical analysis. The data were tested for normality using the Shapiro-Wilk test, and the mean and variance were calculated for normally distributed data. Intergroup differences in BMI, WHR, SBP, and DBP were identified using an independent sample *t* test, and the number of hypertensive patients was determined *via* a chi-square test. $P < 0.05$ indicated statistical differences, and the confidence interval was set at 95%.

RESULTS

Prevalence of DM in the community physical examination population

This study enrolled 37562 community physical examinees, including 4816 (12.82%) elderly people (Figure 1A). Of the 6046 (16.10%) confirmed cases of DM in the total physical examination population, 1575 (26.05%) were elderly DM patients (Figure 1B). Among the 4816 elderly people, 32.70% were diagnosed with DM (Figure 1C). The age of patients in the DM group ranged from 65-81 years, and that of patients in the non-DM group ranged from 67-82 years. Additionally, of the patients in the DM group, 1029 were females and 546 were males. There were 1667 females and 1574 males in the non-DM group. Accordingly, we speculate that DM is a common disease among elderly individuals in the Lugu community.

Comparison of BMI between the two groups

In this study, 4816 elderly people were divided into a DM group and a non-DM group according to their diagnosis of DM, and their BMI was determined. The results showed that the BMI of the individuals in the DM group was 25.16 ± 3.35, while that of the individuals in the non-DM group was 24.61 ± 3.78 (Figure 2A), and these two groups were significantly different.

Comparison of the WHR between the two groups

We also calculated the WHR of both groups and found that the WHR was 0.90 ± 0.04 in the non-DM group and 0.90 ± 0.03 in the DM group, with no significant difference ($P > 0.05$; Figure 2B). Overall, we believe that DM patients have a slightly greater risk of obesity than non-DM patients.

BP analysis of the two groups

The SBP and DBP of the elderly individuals in the two groups were calculated, and the results are shown in Table 1. The left SBP and DBP in the DM group were 137.9 mmHg ± 11.92 mmHg and 69.95 mmHg ± 7.75 mmHg, respectively, while they were 126.6 mmHg ± 12.44 mmHg and 71.15 mmHg ± 12.55 mmHg, respectively, in the non-DM group. The above data revealed significantly greater SBP and lower DBP in the DM group than in the non-DM group.

Prevalence of HT in two groups

In this study, we analyzed the relationship between HT and DM incidence. In the DM group, 1274 patients were

Table 1 Comparison of hypertension and diastolic blood pressure between the two groups

	DM group (*n* = 1575)	Non-DM group (*n* = 3241)	*t* value	*P* value
Systolic blood pressure (left side)	137.90 ± 11.92	126.60 ± 12.44	4.779	< 0.001
Diastolic blood pressure (left side)	69.95 ± 7.75	71.15 ± 12.55	3.475	0.005

DM: Diabetes mellitus; Non-DM: Nondiabetic mellitus.

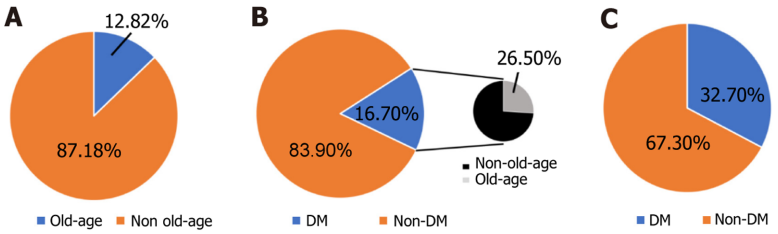

Figure 1 Statistics on the prevalence of diabetes mellitus in the community health examination population. A: The proportion of elderly individuals in the total population who underwent community health examinations; B: The proportion of people with diabetes mellitus (DM) in the community physical examination population; C: The proportion of individuals with DM among the elderly individuals in the community physical examination population. DM: Diabetes mellitus; Non-DM: Nondiabetic mellitus; Nonold-age: Nonelderly; Old-age: Elderly.

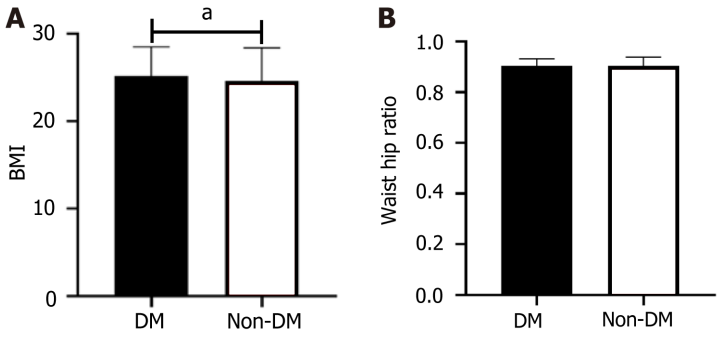

Figure 2 Comparison of body mass index and waist-to-hip ratios between the two groups. A: Body mass index; B: Waist-to-hip ratios. [a]*P* < 0.001. BMI: Body mass index; DM: Diabetes mellitus; Non-DM: Nondiabetic mellitus.

confirmed to have HT, accounting for 80.89%. Among the 3241 non-DM patients, 1743 (53.78%) were hypertensive, and 1498 (46.22%) were nonhypertensive (Table 2). According to the statistical analysis, the number of hypertensive patients in the DM group was much greater than that in the non-DM group ($P < 0.05$). There were more patients with coronary heart disease (CHD) or CKD in the DM group than in the non-DM group ($P < 0.05$) (Table 2). Additionally, there were more patients who drank alcohol more frequently (≥ 3 times) in the DM group than in the non-DM group. Therefore, the results obtained in this study suggested that HT is related to DM among elderly individuals in the Lugu community.

DISCUSSION

The pathogenesis of DM is complicated and involves many factors. For elderly people, the pathophysiological changes caused by aging affect internal metabolic regulation, thus promoting the occurrence of DM; subsequently, aging and DM interact to further promote the progression of diabetic complications; this can also explain the ever-higher incidence of DM among elderly people[15]. A study of atherosclerosis risk based on 5791 older adults revealed that elderly individuals with pre-DM were at a lower risk of death, while those with long-term DM had a higher mortality rate[16], highlighting the importance of timely screening for DM among elderly individuals. In addition, DM is often asymptomatic, and identifying the presence of DM is often difficult until a blood sugar test is performed[17]. Hence, it is necessary to analyze the risk of DM according to other signs. This study included 4816 elderly people to analyze the prevalence of DM and associated risk factors and revealed that HT and high BMI were common in elderly diabetic patients.

The BMI of diabetic elderly individuals was found to be greater than that of nondiabetic elderly individuals. A person can be defined as underweight, normal weight, overweight or obese based on their BMI[18], with a higher BMI indicating a greater degree of fat accumulation. Older people often face the risk of obesity[19], which may further increase the risk of DM. Fat accumulation may be the pathological basis of DM. An obesity environment disrupts the dynamic balance of

Table 2 Statistics of underlying disease in the two groups

	DM group (*n* = 1575)	Non-DM group (*n* = 3241)	χ^2 value	*P* value
			332.9	< 0.001
Hypertension	1274	1743		
Nonhypertension	301	1498		
			227.8	< 0.001
Coronary heart disease	992	1291		
Noncoronary heart disease	583	1950		
			182.4	< 0.001
Chronic kidney disease	986	1357		
Non chronic kidney disease	589	1884		
Alcohol consumption, frequency per week			641.1	< 0.001
< 1 time	198	1569		
1-2 times	529	854		
≥ 3 times	848	818		

DM: Diabetes mellitus; Non-DM: Nondiabetic mellitus.

metabolism, which in turn causes fat accumulation and altered insulin secretion, leading to DM[20]. In contrast, weight loss is associated with improved glycemic control. For elderly individuals whose BMI is close to the warning value or who are already overweight, although they are not currently affected by DM, they should still be urged to control their diet and maintain good exercise habits to return their BMI to normal[21]. It is worth noting that the number of female diabetic patients in this study was higher than that of male patients, which may be related to the local dietary patterns, living habits, and health promotion. Therefore, it is necessary to expand the sample size in subsequent studies to verify the difference in the male-to-female ratio of diabetic patients.

We also noted that the incidence of HT in diabetic patients was greater than that in nondiabetic patients. HT and DM go hand in hand. On the one hand, insulin resistance leads to the destruction of vascular function, resulting in symptoms such as an increase in BP and vascular stiffness; on the other hand, arteriosclerosis and impaired vasodilation contribute to the progression of DM[22]. Research[23] has shown that HT can be an independent predictor of DM and that HT and DM have similar metabolic syndrome phenotypes. Over time, DM also interacts with HT, leading to microvascular and macrovascular lesions and increasing the risk of death[24]. Therefore, HT is an important symptom that cannot be ignored during the progression of DM. Older adults with abnormal BP, especially high BP, need attention before being diagnosed with DM. Additionally, we found that patients with DM have a high risk of CHD and CKD. High-frequency alcohol consumption seems to be related to the occurrence of DM in Lugu Lake.

CONCLUSION

Overall, we believe that older adults in the Lugu community are at high risk of DM, a disease highly correlated with high BMI and HT, CHD and CKD in the elderly population. Therefore, physical examinations should be actively carried out for elderly individuals in the Lugu community to determine their demographic indices, such as BMI, SBP, and DBP. Moreover, adequate attention should be given to those with abnormal signs, and BG levels should be further determined. The findings of this paper provide a valid scientific basis for the health management of elderly individuals in the Lugu community.

ARTICLE HIGHLIGHTS

Research background

The pathogenesis of diabetes mellitus (DM) is complicated and involves many factors. For elderly people, the pathophysiological changes caused by aging affect internal metabolic regulation, thus promoting the occurrence of DM; subsequently, aging and DM interact to further promote the progression of diabetic complications. The aging population and changing lifestyles have made DM more common among older adults.

Research motivation

It is necessary to consider the glycemic indices of the elderly population, focusing on those who may have DM or have been diagnosed. Understanding the prevalence of DM among elderly people in a certain area and discussing the risk factors related to DM are conducive to carrying out targeted interventions for the local elderly population and developing effective blood glucose (BG) management strategies.

Research objectives

To explore the prevalence of DM among elderly people in the Lugu community and analyze the related risk factors to provide a valid scientific basis for the health management of elderly people.

Research methods

A total of 4816 elderly people who came to the community for physical examination were retrospectively analyzed. The prevalence of DM among the elderly was calculated. The individuals were divided into a DM group and a non-DM group according to the diagnosis of DM to compare the differences blood pressure (DBP) in diastolic and systolic blood pressure (SBP), fasting BG (FBG), BMI, waist-to-hip ratio (WHR) and incidence of hypertension (HT), coronary heart disease, and chronic kidney disease (CKD).

Research results

DM was diagnosed in 32.70% of the 4816 elderly people. The BMI of the DM group (25.16 ± 3.35) was greater than that of the non-DM group (24.61 ± 3.78). The WHR was 0.90 ± 0.04 in the non-DM group and 0.90 ± 0.03 in the DM group, with no significant difference. The left SBP and SBP in the DM group were 137.9 mmHg ± 11.92 mmHg and 69.95 mmHg ± 7.75 mmHg, respectively, while they were 126.6 mmHg ± 12.44 mmHg and 71.15 mmHg ± 12.55 mmHg, respectively, in the non-DM group. These findings indicate higher SBP and lower DBP in DM patients than in those without DM. In the DM group, 1274 patients were diagnosed with HT, accounting for 80.89%. Among the 3241 non-DM patients, 1743 (53.78%) were hypertensive and 1498 (46.22%) were nonhypertensive. The DM group had more cases of HT than did the non-DM group. There were more patients with coronary heart disease or CKD in the DM group than in the non-DM group. There were more patients who drank alcohol more frequently (≥ 3 times) in the DM group than in the non-DM group.

Research conclusions

Older adults in the Lugu community are at a higher risk of DM. In the elderly population, DM is closely related to high BMI and HT. Physical examinations should be actively carried out for elderly people to determine their BMI, SBP, DBP, and other signs, and sufficient attention should be given to abnormalities in the above signs before further diagnosis.

Research perspectives

The findings of this paper provide a valid scientific basis for the health management of elderly individuals in the Lugu community.

FOOTNOTES

Author contributions: Zhao LZ designed the research and wrote the first manuscript; Zhao LZ and Li WM contributed to conceiving the research and analyzing data; Zhao LZ and Ma Y conducted the analysis and provided guidance for the research; and all authors reviewed and approved the final manuscript.

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Informed consent statement: Patients were not required to give informed consent to the study because it is a retrospective study and the data came from electronic medical records in the hospital.

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ORIGINAL ARTICLE

Retrospective Study

Influence of blood glucose fluctuations on chemotherapy efficacy and safety in type 2 diabetes mellitus patients complicated with lung carcinoma

Tian-Zheng Fang, Xian-Qiao Wu, Ting-Qi Zhao, Shan-Shan Wang, Guo-Mei-Zhi Fu, Qing-Long Wu, Cheng-Wei Zhou

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Abstract

BACKGROUND

Patients with type 2 diabetes mellitus (T2DM) have large fluctuations in blood glucose (BG), abnormal metabolic function and low immunity to varying degrees, which increases the risk of malignant tumor diseases and affects the efficacy of tumor chemotherapy. Controlling hyperglycemia may have important therapeutic implications for cancer patients.

AIM

To clarify the influence of BG fluctuations on chemotherapy efficacy and safety in T2DM patients complicated with lung carcinoma (LC).

METHODS

The clinical data of 60 T2DM + LC patients who presented to the First Affiliated Hospital of Ningbo University between January 2019 and January 2021 were retrospectively analyzed. All patients underwent chemotherapy and were grouped as a control group (CG; normal BG fluctuation with a mean fluctuation < 3.9 mmol/L) and an observation group (OG; high BG fluctuation with a mean fluctuation ≥ 3.9 mmol/L) based on their BG fluctuations, with 30 cases each. BG-related indices, tumor markers, serum inflammatory cytokines and adverse reactions were comparatively analyzed. Pearson correlation analysis was

performed to analyze the correlation between BG fluctuations and tumor markers.

RESULTS

The fasting blood glucose and 2-hour postprandial blood glucose levels in the OG were notably elevated compared with those in the CG, together with markedly higher mean amplitude of glycemic excursions (MAGE), mean of daily differences, largest amplitude of glycemic excursions and standard deviation of blood glucose ($P < 0.05$). In addition, the OG exhibited evidently higher levels of carbohydrate antigen 19-9, carbohydrate antigen 125, carcinoembryonic antigen, neuron-specific enolase, cytokeratin 19, tumor necrosis factor-α, interleukin-6, and high-sensitivity C-reactive protein than the CG ($P < 0.05$). Pearson analysis revealed a positive association of MAGE with serum tumor markers. The incidence of adverse reactions was significantly higher in the OG than in the CG ($P < 0.05$).

CONCLUSION

The greater the BG fluctuation in LC patients after chemotherapy, the more unfavorable the therapeutic effect of chemotherapy; the higher the level of tumor markers and inflammatory cytokines, the more adverse reactions the patient experiences.

Key Words: Blood glucose fluctuation; Type 2 diabetes mellitus; Lung carcinoma; Tumor markers

Core Tip: Controlling hyperglycemia may have important therapeutic implications for cancer patients. In this study, we seek to clarify the influence of blood glucose fluctuations on chemotherapy efficacy and safety in type 2 diabetes mellitus patients complicated with lung carcinoma.

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INTRODUCTION

Lung carcinoma (LC) is the most common malignant tumor in clinical practice that seriously harms human health, with the distinct characteristics of high incidence, high fatality rate and difficult treatment[1,2]. Diabetes mellitus (DM) is a common chronic metabolic disorder, with type 2 DM (T2DM) being the most prevalent. The prevalence of DM complicated with LC is increasing worldwide[3,4]. Epidemiological studies have shown that people with DM have a higher risk of LC[5,6], possibly because of common risk factors between the two diseases[7]. For example, smoking, a major external factor of LC, has also been indicated to have a certain relationship with DM[8,9]. In addition, the incidence of both diseases is correlated with age, with the highest prevalence found in middle-aged and elderly people among all age groups[10]. An estimated 8% to 18% of patients with non-small cell LC (NSCLC) have been reported to have DM[11]. DM may contribute to LC progression through mechanisms such as hyperinsulinemia, hyperglycemia and chronic inflammation, which are related to cell proliferation and cancer progression[12]. LC complicated with DM can obviously involve elevated blood glucose (BG) levels in patients, which causes the patients' body to be always in a state of injury. The immunity of such patients with large BG fluctuations will be reduced to a certain extent, resulting in abnormal metabolic function[13]. In addition, diabetes is associated with a 42 percent increased risk of death, a 21 percent elevated risk of recurrence, and significantly lower 5-year overall and cancer-specific survival[14-16]. Therefore, proper management of comorbid DM is essential for cancer treatment.

Chemotherapy is most commonly used clinically for LC with concomitant T2DM but no dominant locus mutations [17]. Although chemotherapy can partially control the tumor, DM and cancer patients undergoing chemotherapy are at an increased risk of developing BG problems due to the influence of metabolism and blood glucose, resulting in poor overall clinical efficacy and adverse prognosis[18,19]. Hyperglycemia in cancer patients receiving chemotherapy is related to the risk of nonhematological toxicity[20]. The combination of chemotherapy and corticosteroids, commonly used in cancer treatment, puts patients at risk of developing hyperglycemia, a clinical toxicity that may affect the reduction, interruption or cessation of chemotherapy doses[21]. Therefore, BG fluctuation is definitely a factor affecting the chemotherapy efficacy and prognosis of patients with DM complicated with cancer. However, whether the BG level can be a predictor of chemotherapy efficacy in LC patients has rarely been studied, and epidemiological evidence is limited. Accordingly, this study focuses on the influence of BG fluctuations on chemotherapy efficacy in T2DM + LC patients.

MATERIALS AND METHODS

Study participants

The clinical data of 60 T2DM + LC patients treated in the First Affiliated Hospital of Ningbo University from January 2019 to January 2021 were retrospectively analyzed. All patients were treated with chemotherapy. The inclusion criteria were as follows: (1) Patients meeting the diagnostic criteria for T2DM and pathologically confirmed with LC; (2) Patients with an estimated life expectancy exceeding 6 months; (3) Patients with no other vital organ function diseases; (4) Patients with no drug interactions; (5) Patients with normal heart, liver and kidney function; and (6) patients with complete clinical and follow-up data. The exclusion criteria were as follows: (1) Type 1 DM or secondary DM; (2) Renal and liver failure; (3) Mental illness or infectious diseases; and (4) Incomplete clinical and follow-up data. According to BG fluctuations, patients were assigned to normal [control group (CG); mean BG fluctuation < 3.9 mmol/L] and high blood BG range groups [observation group (OG); mean BG fluctuation ≥ 3.9 mmol/L], each with 30 cases. The male-to-female ratio, average age, and mean duration of DM in the CG were 16:14, 59.40 ± 3.00 years, and 2.27 ± 1.10 years, respectively, while those in the OG were 18:12, 59.17 ± 2.57 years, and 2.75 ± 1.22 years, respectively. Patients in the OG and CG were not significantly different in general data and were clinically comparable ($P > 0.05$).

Methods

All patients completed chemotherapy and were continuously monitored for BG fluctuations through a dynamic BG monitoring system. Forty-eight hours later, glycemic fluctuation indices, including the mean amplitude of glycemic excursions (MAGE), mean of daily differences (MODD), largest amplitude of glycemic excursions (LAGE), and standard deviation of BG (SDBG), were read. All patients remained on an empty stomach for more than 10 h prior to the examination and had elbow venous blood drawn early the next morning for testing. BG-related indices [fasting BG (FBG) and 2-h postprandial BG (2Hpg)] were detected using an automatic biochemical analyzer. Tumor markers, including carbohydrate antigen 19-9 (CA19-9), CA125, carcinoembryonic antigen (CEA), neuron-specific enolase (NSE), and cytokeratin 19 (CYFRA21-1), were determined with the use of an automatic chemiluminescence immunoassay. Turbidimetric immunoassay was also performed for the measurement of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and high-sensitivity C-reactive protein (hs-CRP). The occurrence of adverse reactions in the two groups of patients, including phlebitis, gastrointestinal reactions, oral ulcers, liver and kidney damage, *etc*., was observed.

Endpoints

The primary endpoints were the changes in serum tumor markers in the two groups, while the secondary endpoints were glycemic fluctuation indices, changes in inflammatory factors, and adverse reactions.

Statistical analysis

The data were statistically analyzed by SPSS 25.0. Continuous (expressed by mean and standard deviation) and categorical variables (represented by percentages) were analyzed by the t test and χ^2 test, respectively. The correlation between serum tumor markers and MAGE was identified by Pearson analysis at an $\alpha = 0.05$ Level of significance. A P value < 0.05 was considered significant for all tests.

RESULTS

Glycemic fluctuation indices after chemotherapy in the two groups

FBG and 2hPG in the OG were significantly higher than those in the CG ($P < 0.05$), as shown in Figure 1. In addition, the OG exhibited markedly higher MAGE, MODD, LAGE, and SDBG than the CG ($P < 0.05$; Table 1). These results indicated that in patients with high fluctuating blood glucose ranges have relatively weaker glycemic control and greater blood glucose fluctuations after chemotherapy.

Levels of tumor markers after chemotherapy in the two groups

After chemotherapy, patients in both groups were tested to compare the levels of tumor markers. CA19-9, CA125, CEA, NSE and CYFRA21-1 Levels were found to be significantly higher in the RG than in the CG ($P < 0.05$), as shown in Table 2. The results similarly implied that patients with blood glucose in the normal range of fluctuation have more stable tumor marker levels after chemotherapy.

Serum levels of inflammatory cytokines in the two groups

Statistical significance was determined in serum TNF-α, IL-6 and hs-CRP levels between groups, with even higher levels of these inflammatory cytokines in the OG ($P < 0.05$; Figure 2). These results indicated that high levels of blood glucose fluctuations impair the body's immune function, resulting in abnormalities in the body's immune defense system, various types of inflammation, and a decrease in the ability of white blood cells to act, producing large amounts of inflammatory factors.

Correlation between BG fluctuations and tumor marker levels

According to Pearson analysis (Figure 3), MAGE was positively associated with serum CA19-9 ($r = 0.4724$, $P = 0.0001$), CA125 ($r = 0.5508$, $P < 0.0001$), CEA ($r = 0.5441$, $P < 0.0001$), NSE ($r = 0.5719$, $P < 0.0001$), and CYFRA21-1 ($r = 0.6425$, $P <$

Table 1 Comparison of glycemic fluctuation indices after chemotherapy

	MAGE (mmol/L)	MODD (mmol/L)	LAGE (mmol/L)	SDBG (mmol/L)
Control group (n = 30)	3.75 ± 1.07	2.06 ± 0.36	4.25 ± 0.90	2.13 ± 0.33
Observation group (n = 30)	5.61 ± 1.15	2.59 ± 0.51	6.31 ± 1.21	2.72 ± 0.40
t value	6.451	4.721	7.472	6.195
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001

MAGE: Mean amplitude of glycemic excursions; MODD: Mean of daily differences; LAGE: Largest amplitude of glycemic excursions; SDBG: Standard deviation of blood glucose.

Table 2 Comparison of tumor markers after chemotherapy

	CA19-9 (U/mL)	CA125 (U/mL)	CEA (mg/L)	NSE (mg/L)	CYFRA21-1 (mg/L)
Control group (n = 30)	64.26 ± 5.20	65.27 ± 6.60	12.12 ± 2.11	15.97 ± 1.34	4.98 ± 0.82
Observation group (n = 30)	75.00 ± 9.48	83.72 ± 6.06	17.83 ± 2.28	19.86 ± 1.42	7.54 ± 0.96
t value	5.444	11.28	10.08	10.88	11.11
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; NSE: Neuron-specific enolase; CYFRA21-1: Cytokeratin 19.

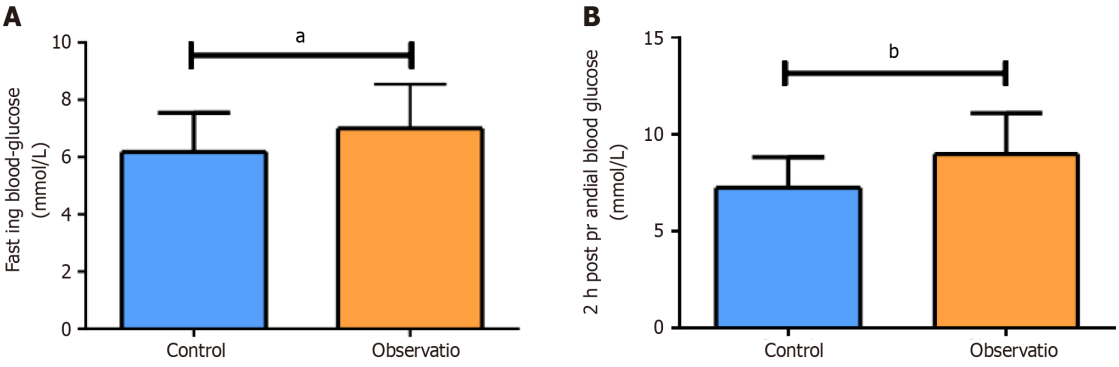

Figure 1 Comparison of blood glucose levels after chemotherapy. A: Fasting blood glucose level of the two groups after chemotherapy; B: 2-h postprandial blood glucose level of the two groups after chemotherapy. [a]P < 0.05, [b]P < 0.001.

0.0001).

Adverse reactions in the two groups

The incidence of phlebitis, gastrointestinal reactions, oral ulcers, and liver and kidney dysfunction in the OG was 33.3%, 50.0%, 36.7%, and 26.7%, respectively, the values of which were significantly higher than those in the CG (10.0%, 20.0%, 13.3%, and 6.7%, respectively) (P < 0.05; Table 3). These results indicated that hyperglycemia can also cause adverse reactions such as gastrointestinal reactions, liver damage, kidney damage, and oral ulcers, resulting in reduced safety.

DISCUSSION

There are abnormalities in insulin secretion in patients with T2DM, which leads to a compensatory increase in insulin and a gradual increase in insulin content in blood[22]. Research has linked the occurrence of LC to the specific and nonspecific immunity of diabetic patients, so many experts believe that T2DM will lead to an increase in the incidence of LC[23]. At the same time, in clinical LC research, the frequency of comorbidities in LC patients is very high, of which DM is the most common[24]. During chemotherapy for T2DM with LC, poor glycemic control is often associated with a more clinically aggressive cancer course and occurrence of adverse events such as neutropenia, infection, and death[25,26]. However, chemotherapy itself can lead to abnormal glycolipid metabolism in cancer patients[27]. Glucose metabolism disorders occur after chemotherapy, leading to a significant increase in blood glucose values and even diabetes. It will suspend

Table 3 Incidence of adverse reactions in the two groups of patients, *n* (%)

	Phlebitis	Gastrointestinal reactions	Oral ulcers	Liver and kidney dysfunction
Control group (n = 30)	3 (10.0)	6 (20.0)	4 (13.3)	2 (6.7)
Observation group (n = 30)	10 (33.3)	15 (50.0)	11 (36.7)	8 (26.7)
t value	4.8121	5.9341	4.3561	4.3201
P value	0.0283	0.0149	0.0369	0.0377

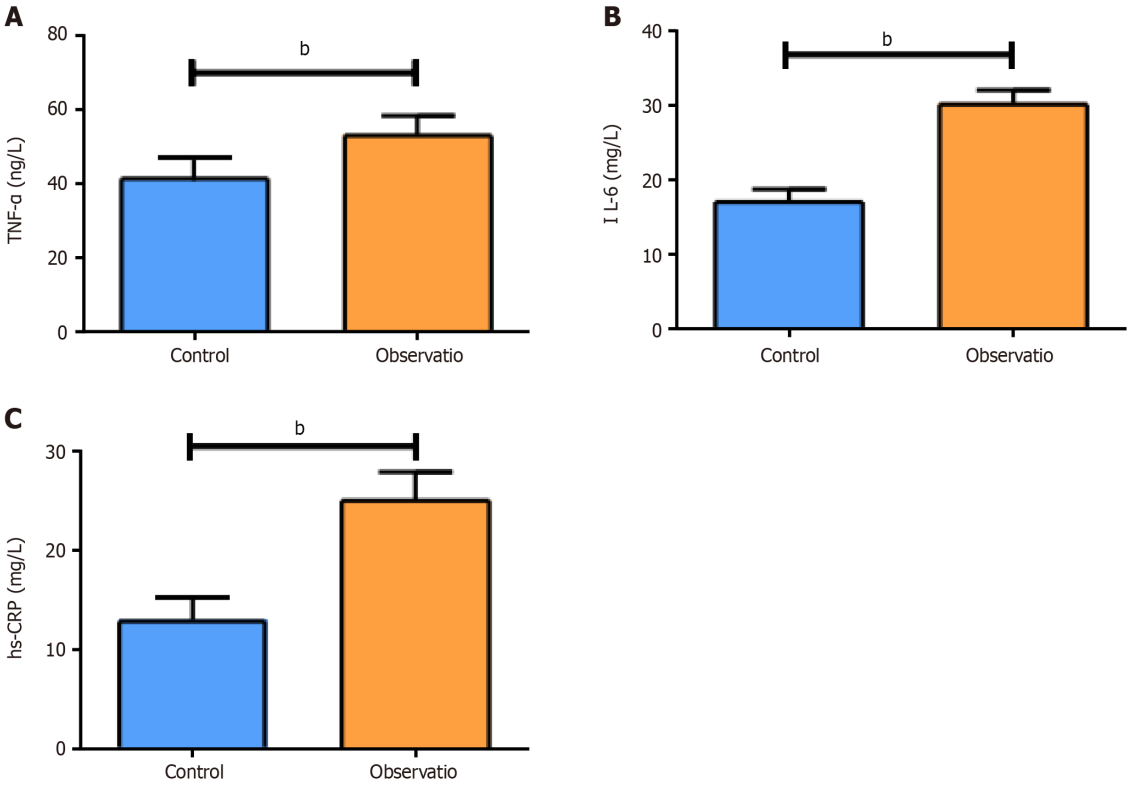

Figure 2 Comparison of serum inflammatory cytokines after chemotherapy. A: Tumor necrosis factor-α level of the two groups after chemotherapy; B: Interleukin-6 Level of the two groups after chemotherapy; C: High-sensitivity C-reactive protein level of the two groups after chemotherapy. $^{b}P < 0.001$. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; hs-CRP: High-sensitivity C-reactive protein.

chemotherapy and affect the quality of life of patients[28]. Therefore, the control of BG fluctuations occupies an important position in T2DM + LC patients undergoing chemotherapy, which can affect the curative effect of chemotherapy.

BG fluctuations refer to the amplitude of glycemic excursions between the highest and the lowest BG levels over time. There are also BG fluctuations in healthy people, but the amplitude is small, being mostly due to invalid fluctuations[29]. Abnormal BG fluctuations in diabetic patients can easily aggravate abnormalities in islet B cells, which affects physiological processes such as insulin cell apoptosis and causes abnormal endothelial cell proliferation, triggering vascular endothelial dysfunction[30]. MAGE is a "golden indicator" reflecting BG fluctuations[31]. In this study, the patients were assigned to normal (CG) and high (OG) BG fluctuation groups according to BG fluctuation amplitude. The results showed that MAGE, MODD, LAGE, SDBG, FBG and 2hPG in the OG were significantly higher than those in the CG. High-level BG indices indicate the provision of sufficient nutrients and other necessary conditions for the infinite division and proliferation of tumor cells. BG fluctuation measurements in NSCLC patients showed that patients with large glycemic variability and high BG levels had poor prognosis and increased mortality and disability[32]. Also, Hyperglycemia reduces the response to chemotherapeutic drugs, directly affects tumor cell growth, and induces drug resistance in tumor cells[18]. Meanwhile, long-term abnormal BG fluctuations or hyperglycemia in LC patients can aggravate the degree of oxidative stress in vivo, activate the protein kinase C pathway, and further promote vascular endothelial cell apoptosis and endothelial cell DNA oxidative damage, leading to an increase in tumor markers such as CA128, CA19-9 and CEA. In this study, markedly higher CA19-9, CA125, CEA, NSE and CYFRA21-1 Levels were determined in the OG than in the CG. In addition, the levels of inflammatory factors in the OG also increased significantly. Of these, hs-CRP is an acute-phase protein, and its level can be significantly increased when the body is disturbed by inflammation[33]. TNF-α is an important inflammatory index closely related to vascular endothelial injury and coagulation state[34]. The decrease in insulin secretion in T2DM + LC patients can lead to increased levels of insulin antibodies and abnormal metabolism of proteins, fats and sugars, increasing blood viscosity and BG, causing microcircu-

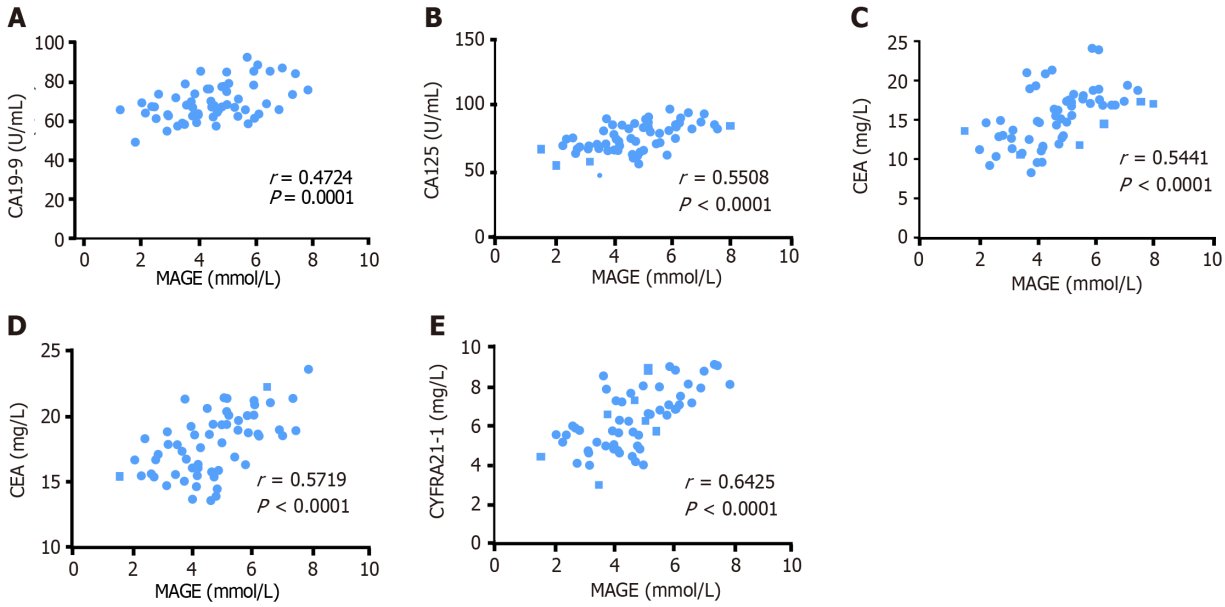

Figure 3 Correlation between mean amplitude of glycemic excursions and tumor markers in all patients. A: Correlation of mean amplitude of glycemic excursions (MAGE) with carbohydrate antigen 19-9 (CA19-9) level in all patients; B: Correlation of MAGE with CA125 level in all patients; C: Correlation of MAGE with carcinoembryonic antigen level in all patients; D: Correlation of MAGE with neuron-specific enolase level in all patients; E: Correlation of MAGE with cytokeratin 19 level in all patients. MAGE: Mean amplitude of glycemic excursions; CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; NSE: Neuron-specific enolase; CYFRA21-1: Cytokeratin 19.

lation disorder and affecting tissue defense function. As such, the body's humoral and cellular immunity is reduced, leukocyte function is weakened, and inflammatory reactions are induced, damaging the pulmonary vascular barrier, generating oxygen free radicals, and accelerating cancer cell proliferation[35]. Through Pearson correlation analysis, MAGE was determined to be positively correlated with serum tumor markers, further confirming the influence of BG fluctuation amplitude on the chemotherapy efficacy of patients. Finally, we observed the adverse reactions of both groups of patients. Long-term hyperglycemia in the body will also promote the generation of oxidative stress, which will lead to gastrointestinal reactions, liver damage, kidney damage and oral ulcers, resulting in reduced safety. The results also confirmed an obviously higher incidence of adverse reactions in patients with larger BG fluctuations than in patients with normal BG fluctuations. Therefore, it is of great importance to control hyperglycemia in cancer patients to control disease progression.

However, this study still has some limitations. Limitations of this study include the small clinical sample size and retrospective nature, so case selection bias may be encountered. Also, survival information after chemotherapy in oncology patients at risk for high magnitude of glycemic fluctuations has not been analyzed, and further research is needed to investigate the relationship between glycemic control and adverse outcomes. Thus, a multiple center, large sample size and prospective study is need to further investigate the relationship between blood glucose levels and cancer treatment efficacy.

CONCLUSION

Taken together, large BG fluctuations can enhance the levels of tumor markers and inflammatory factors in T2DM + LC patients and inhibit chemotherapy efficacy, with low safety. Therefore, in such patients, the BG indicators should be strictly controlled clinically to ensure prognosis.

ARTICLE HIGHLIGHTS

Research background

Lung carcinoma (LC) is the most common malignant tumor in clinical practice that seriously harms human health. Diabetes mellitus (DM) is a common chronic metabolic disorder, with type 2 DM (T2DM) being the most prevalent. The prevalence of DM complicated with LC is increasing worldwide.

Research motivation

Blood glucose (BG) fluctuation is definitely a factor affecting the chemotherapy efficacy and prognosis of patients with DM complicated with cancer. However, whether the BG level can be a predictor of chemotherapy efficacy in LC patients

has rarely been studied, and epidemiological evidence is limited.

Research objectives
This study focuses on the influence of BG fluctuations on chemotherapy efficacy in T2DM + LC patients.

Research methods
The clinical data of 60 T2DM + LC patients were retrospectively analyzed. All patients underwent chemotherapy and were grouped as a control group and an observation group based on their BG fluctuations, with 30 cases each. BG-related indices, tumor markers, serum inflammatory cytokines and adverse reactions were comparatively analyzed.

Research results
After chemotherapy, fasting BG and 2-h postprandial BG in the observation group were significantly higher than those in the control group. In addition, the observation group exhibited markedly higher mean amplitude of glycemic excursions, mean of daily differences, largest amplitude of glycemic excursions, and standard deviation of BG than the control group patients with high fluctuating blood glucose ranges have relatively weaker glycemic control and greater blood glucose fluctuations after chemotherapy. The observation group has higher levels of tumor markers and inflammatory indicators than the control group, as well as adverse event rate.

Research conclusions
Large BG fluctuations can enhance the levels of tumor markers and inflammatory factors in T2DM + LC patients and inhibit chemotherapy efficacy, with low safety.

Research perspectives
The control of BG fluctuations occupies an important position in T2DM + LC patients undergoing chemotherapy, which can affect the curative effect of chemotherapy.

FOOTNOTES

Co-first authors: Tian-Zheng Fang and Xian-Qiao Wu.

Author contributions: Fang TZ and Wu XQ contributed equally to this work and are co-first authors; Fang TZ, Wu XQ and Zhou CW conceived and designed the study; Fang TZ, Wu XQ, Zhao TQ, Wang SS, Fu GMZ, Wu QL and Zhou CW guided the study; Fang TZ, Wu XQ collected the clinical date; Fang TZ, Wu XQ and Zhou CW analyzed the data; and all authors drafted and revised the manuscript.

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Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ORIGINAL ARTICLE

Retrospective Study

Construction and validation of a neovascular glaucoma nomogram in patients with diabetic retinopathy after pars plana vitrectomy

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Abstract

BACKGROUND

Neovascular glaucoma (NVG) is likely to occur after pars plana vitrectomy (PPV) for diabetic retinopathy (DR) in some patients, thus reducing the expected benefit. Understanding the risk factors for NVG occurrence and building effective risk prediction models are currently required for clinical research.

AIM

To develop a visual risk profile model to explore factors influencing DR after surgery.

METHODS

We retrospectively selected 151 patients with DR undergoing PPV. The patients were divided into the NVG (NVG occurrence) and No-NVG (No NVG occurrence) groups according to the occurrence of NVG within 6 months after surgery. Independent risk factors for postoperative NVG were screened by logistic regression. A nomogram prediction model was established using R software, and the model's prediction accuracy was verified internally and externally, involving the receiver operator characteristic curve and correction curve.

RESULTS

After importing the data into a logistic regression model, we concluded that a posterior capsular defect, preoperative vascular endothelial growth factor ≥ 302.90 pg/mL, glycosylated hemoglobin ≥ 9.05%, aqueous fluid interleukin 6 (IL-6) ≥ 53.27 pg/mL, and aqueous fluid IL-10 ≥ 9.11 pg/mL were independent risk factors for postoperative NVG in patients with DR ($P < 0.05$). A nomogram model was established based on the aforementioned independent risk factors, and a computer simulation repeated sampling method was used to internally and externally verify the nomogram model. The area under the curve (AUC), sensitivity, and specificity of the model were 0.962 [95% confidence interval (95%CI): 0.932-0.991], 91.5%, and 82.3%, respectively. The AUC, sensitivity, and specificity of the external validation were 0.878 (95%CI: 0.746-0.982), 66.7%, and 95.7%, respectively.

CONCLUSION

A nomogram constructed based on the risk factors for postoperative NVG in patients with DR has a high prediction accuracy. This study can help formulate relevant preventive and treatment measures.

Key Words: Diabetic retinopathy; Retinopathy; Neovascular; Glaucoma; Risk factors; Nomogram

Core Tip: The primary treatment for diabetic retinopathy (DR) is pars plana vitrectomy (PPV); however, neovascular glaucoma (NVG) is likely to occur after surgery. This affects the prognosis of surgery. Risk factors for NVG after PPV have been studied; however, whether inflammatory factors in the aqueous humor are related to the risk of NVG formation is unknown. We explored the risk factors (including inflammatory factors) for NVG and built a histogram model based on these factors, which confirmed the effectiveness and applicability of this model in assessing NVG after PPV in patients with DR.

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INTRODUCTION

Diabetic retinopathy (DR) is a clinical complication of diabetes, and its fundus manifestations include retinal exudation, edema, angiogenesis, hemorrhage, and proliferative membrane formation[1]. The primary treatment is pars plana vitrectomy (PPV), which effectively controls disease progression[2]. However, some patients develop postoperative neovascular glaucoma (NVG). NVG is a secondary glaucoma that can lead to severe visual impairment or even complete blindness, seriously affecting the postoperative recovery and prognosis of patients[3,4]. Medical researchers widely believe that NVG is closely related to the release of various cytokines induced by retinal ischemia and hypoxia, which promotes extensive neovascularization and causes ocular hypertension by blocking the anterior chamber horn[5]. Relevant reports have shown that inflammatory regulators are closely related to DR neovascularization[6]. There is an urgent need to explore the relevant factors of postoperative NVG in DR and improve the postoperative recovery and prognostic effects of patients with DR. Currently, there are few reports on integrating the relevant factors of postoperative NVG in patients with DR and building risk screening tools based on this[7-9]. A nomogram model can visualize the results of multifactor analysis and be intuitively used to predict individual risk factors[10]. In this study, a nomogram was constructed to predict postoperative NVG in DR, providing a theoretical basis for the clinical screening of high-risk groups and the formulation of relevant preventive measures.

MATERIALS AND METHODS

Patients

We retrospectively selected 151 patients with DR who had undergone PPV at the Tianjin Medical University Eye Hospital. The treatment period for the selected patients was between January 2019 and December 2020. Patients were enrolled in the NVG group (with NVG) and the No-NVG group (without NVG) according to the occurrence of NVG within 6 months after surgery. The included patients met the following conditions: (1) Clinical diagnosis of DR and PPV treatment; and (2) complete information at the 6-month follow-up. The exclusion criteria were as follows: (1) Prior history of glaucoma or ocular hypertension; and (2) recurrent vitreous hemorrhage.

Method

The clinical data of the patients were collected. They included demographic data (age, sex, and body mass) at admission, clinical information [type of diabetes, DR severity, duration of retinopathy, hypertensive or not, whether hyperlipidemic, preoperative intraocular pressure, duration of surgery, number of vitrectomies performed, intraocular fillings, whether combined cataract surgery was performed, whether ipsilateral carotid artery stenosis was ≤ 25.0%, residual in retinal non-perfusion area, preoperative anti-vascular endothelial growth factor (VEGF) therapy], and preoperative laboratory data [serum VEGF, glycosylated hemoglobin (HbAlc), interleukin (IL)-6] and IL-10 in serum and aqueous humor).

Carotid artery stenosis assessment was as follows: Carotid intima-media thickness detected by color Doppler ultrasound of < 1.0 mm, 11.2 mm, 1.2-1.4 mm, and > 1.4 mm were categorized as normal, intimal thickening, plaque formation, and carotid artery stenosis, respectively. The degree of carotid artery stenosis with reference to blood flow velocity and whether the carotid artery stenosis of patients was ≤ 25.0% was assessed.

Fundus fluorescein angiography and optical coherence tomography angiography were used to determine the boundary of the non-perfusion area. After correcting the scale in the fundus fluorescein angiography image, Image J software version 1.48 measured the non-perfusion area.

Five mL of fasting venous blood was extracted from the patient and stored in a -70 °C refrigerator for examination. Serum VEGF was detected by enzyme-linked immunosorbent assay (ELISA). The kit was purchased from Shanghai Renjie Biotechnology Co., LTD. HbA1C was determined by high-performance liquid chromatography using Gimp LC-4000 high-performance liquid chromatography. Serum IL-6, IL-10, and tumor necrosis factor (TNF-α) were detected by biotin-avidin double antibody sandwich ELISA.

Establishment and verification of the risk nomogram model

Demographic, clinical, and preoperative laboratory data of patients with and without NVG were compared. We incorporated variables with statistically significant differences into the logistic regression model to identify the risk factors for NVG. The rms package of the R language (R 4.0.3) software was used to establish a nomogram model for the risk of postoperative NVG in patients with DR. The line diagram model was internally verified using bootstrap sampling 500 times. The clinical and related laboratory data of another 72 patients (including 12 patients with NVG; the incidence of NVG was 16.67%) who underwent PPV (between January 2021 and December 2021) were used as an external validation cohort based on the same inclusion and exclusion criteria. After internal and external validation, we evaluated the differentiation using the receiver operator characteristic curve (ROC). A calibration curve was used to evaluate the degree of nomogram calibration.

Statistical processing

Statistical software (SPSS 23.0) was used to analyze the data. Qualitative data are presented as frequencies and percentages, and the two groups were compared using the chi-square test. The continuous correction chi-square was adopted when 1 ≤ theoretical frequency < 5, and the total sample size was ≥ 40. Quantitative normal distribution data are presented as means ± SD, and we compared the groups using a *t*-test. Additionally, quantitative non-normal distribution data were described as M (P25, P75) and analyzed using the Mann-Whitney *U* test. Risk factors were analyzed using binary logistic regression. The test level is $\alpha = 0.05$.

RESULTS

Differences in clinical baseline indicators

Among the 151 patients with DR who underwent PPV, 21 (13.91%) developed NVG (NVG group), and 130 (No-NVG group) did not develop NVG within 6 months after surgery. Compared with the No-NVG group, the ratio of posterior capsular defect, ipsilateral carotid artery stenosis ≤ 25.0%, residual retinal non-perfusion area, and the levels of preoperative VEGF, HbAlc, IL-6, IL-10, and TNF-α in aqueous humor were higher in the NVG group during cataract surgery ($P < 0.05$) (Table 1).

Analysis of factors affecting postoperative NVG in patients with DR

With the occurrence of NVG (1 = occurrence, 0 = non-occurrence) in patients with DR after surgery as the dependent variable, eight factors with statistical significance in univariate analysis (posterior capsular integrity in combined cataract surgery, ipsilateral carotid artery stenosis ≤ 25.0%, residual retinal non-perfusion area, preoperative VEGF, HbAlc, aqueous humor IL-6, aqueous humor IL-10, and aqueous humor TNF-α) were used as independent variables. Original values of measurement data were entered and assigned to classified data [integrity of posterior capsule (1 = defect, 0 = integrity), ipsilateral carotid artery stenosis ≤ 25.0% (1 = yes, 0 = no), and residual retinal non-perfusion area (1 = yes, 0 = no)]. Multivariate logistic regression analysis showed that a posterior capsular defect, preoperative VEGF ≥ 302.90 pg/mL, HbAlc ≥ 9.05%, aqueous fluid IL-6 ≥ 53.27 pg/mL, and aqueous fluid IL-10 ≥ 9.11 pg/mL in combined cataract surgery were independent risk factors for postoperative NVG in patients with DR ($P < 0.05$; Table 2).

Establishment of a risk model for postoperative NVG profile in patients with DR

Based on the results of logistic regression analysis, R software was used to construct a nomogram model for predicting NVG risk (Figure 1). Based on the column nomogram, NVG risk can be quickly predicted. A patient with a posterior capsular defect and preoperative VEGF = 250 pg/mL, HbAlc = 10%, aqueous fluid IL-6 = 60 pg/mL, and IL-10 = 13.5 pg/

Table 1 Differences in clinical data between the neovascular glaucoma and No- neovascular glaucoma-groups

Data		NVG group (*n* = 21)	No-NVG group (*n* = 130)	χ^2/t	*P* value
Sex, *n* (%)	Male	12 (57.14)	78 (60.00)	0.061	0.084
	Female	9 (42.86)	52 (40.00)		
Age (mean ± SD, yr)		57.58 ± 8.12	55.21 ± 10.27	1.007	0.315
BMI (mean ± SD, kg/m^2)		22.28 ± 3.16	22.24 ± 3.41	0.050	0.959
Diabetes duration (mean ± SD, yr)		10.23 ± 3.11	10.37 ± 3.46	0.174	0.861
Diabetes type, *n* (%)	Type 1	4 (19.05)	11 (8.46)	1.236	0.266
	Type 2	17 (80.95)	119 (91.54)		
DR severity	Nonproliferative	8 (38.10)	64 (49.23)	0.899	0.343
	Proliferative	13 (61.90)	66 (50.77)		
Complicated with hypertension, *n* (%)	Yes	14 (66.67)	59 (45.38)	3.279	0.070
	No	7 (33.33)	71 (54.62)		
Combined hyperlipidemia, *n* (%)	Yes	13 (61.90)	61 (46.92)	1.624	0.203
	No	8 (38.10)	69 (53.08)		
Preoperative intraocular pressure (mean ± SD, mmHg)		15.23 ± 2.81	15.26 ± 2.87	0.044	0.964
Operation time (mean ± SD, min)		125.42 ± 16.85	120.13 ± 15.73	1.416	0.158
Intraocular pressure 7 d after surgery (mean ± SD, mmHg)		19.15 ± 2.25	18.16 ± 2.41	1.762	0.080
Number of vitrectomies, *n* (%)	First time	15 (71.43)	110 (84.62)	2.206	0.138
	Not the first time	6 (28.57)	20 (15.38)		
Intraocular filler, *n* (%)	Silicone oil	3 (14.29)	38 (29.23)	1.356	0.244
	BSS or gas fill	18 (85.71)	92 (70.77)		
Combined cataract surgery, *n* (%)	Yes	12 (57.14)	52 (40.0)	2.176	0.140
	No	9 (42.86)	78 (60.0)		
Posterior capsular integrity in combined cataract surgery, *n* (%)	Complete	15 (71.43)	128 (98.46)	6.441	< 0.001
	Defect	6 (28.57)	12 (9.23)		
Ipilateral carotid artery stenosis ≤ 25.0%, *n* (%)	Yes	4 (19.05)	6 (4.62)	6.090	< 0.001
	No	17 (80.95)	124 (95.38)		
Residual retinal non-perfusion area, *n* (%)	Yes	7 (33.33)	17 (13.08)	5.549	< 0.001
	No	14 (66.67)	113 (86.92)		
Preoperative anti-VEGF drug therapy, *n* (%)	Yes	3 (14.29)	42 (32.31)	2.012	0.156
	No	18 (85.71)	88 (67.69)		
Preoperative VEGF (mean ± SD, pg/mL)		312.01 ± 29.29	275.64 ± 36.92	4.297	< 0.001
HbA1C (mean ± SD, %)		9.31 ± 1.29	8.42 ± 1.07	3.434	< 0.001
Serum IL-6 (mean ± SD, pg/mL)		3.51 ± 0.67	3.15 ± 0.85	1.848	0.066
Serum IL-10 (mean ± SD, pg/mL)		2.62 ± 0.42	2.74 ± 0.57	0.923	0.357
Serum TNF-α (mean ± SD, pg/mL)		10.88 ± 3.41	9.52 ± 3.06	1.860	0.064
Aqueous IL-6 (mean ± SD, pg/mL)		54.54 ± 15.81	39.14 ± 11.10	5.526	< 0.001
Aqueous IL-10 (mean ± SD, pg/mL)		9.91 ± 2.77	7.94 ± 1.92	4.067	< 0.001
Aqueous TNF-α (mean ± SD, pg/mL)		5.00 ± 1.26	3.91 ± 0.75	5.499	< 0.001

NVG: Neovascular glaucoma; BMI: Body mass index; DR: Diabetic retinopathy; VEGF: Vascular endothelial growth factor; HbAlc: Glycosylated hemoglobin; IL-6: Interleukin-6; IL-10: Interleukin-10; TNF-α: Tumor necrosis factor.

Table 2 Logistic regression analysis of postoperative neovascular glaucoma in patients with diabetic retinopathy

Variables	β	SE	Wald χ^2	*P* value	OR (95%CI)
Posterior capsular integrity in combined cataract surgery	2.474	1.045	5.608	0.018	11.868 (1.532-91.953)
Ipilateral carotid artery stenosis ≤ 25.0%	1.202	1.33	0.817	0.366	3.328 (0.245-45.148)
Residual retinal non-perfusion area	1.505	1.087	1.918	0.166	4.504 (0.535-37.904)
Preoperative VEGF	0.047	0.018	6.844	0.009	1.048 (1.012-1.086)
HbA1c	0.689	0.267	6.678	0.010	1.992 (1.181-3.361)
Aqueous IL-6	0.101	0.036	8.135	0.004	1.107 (1.032-1.186)
Aqueous IL-10	0.756	0.240	9.959	0.002	2.130 (1.332-3.406)
Aqueous TNF-α	0.304	0.466	0.426	0.514	1.356 (0.543-3.382)
Constant	-35.565	8.211	18.76	< 0.001	-

NVG: Neovascular glaucoma; DR: Diabetic retinopathy; VEGF: Vascular endothelial growth factor; HbAlc: Glycosylated hemoglobin; IL-6: Interleukin-6; IL-10: Interleukin-10; TNF-α: Tumor necrosis factor alpha.

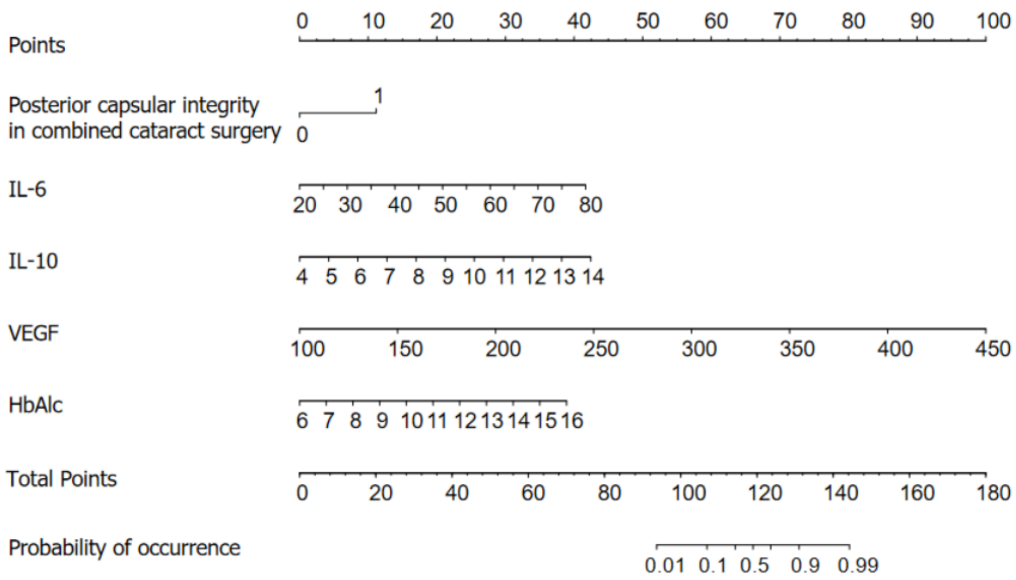

Figure 1 A nomogram model predicting the risk of neovascular glaucoma in patients with diabetic retinopathy After surgery. IL-6: Interleukin-6; IL-10: Interleukin-10; VEGF: Vascular endothelial growth factor; HbAlc: Glycosylated hemoglobin; NVG: Neovascular glaucoma; DR: Diabetic retinopathy.

mL, during combined cataract surgery had a total score of 133.5 (11.0 + 42.0 + 15.0 + 27.5 + 40.0), suggesting a 90% risk of postoperative NVG.

Internal validation of the nomogram model

The area under the ROC curve (AUC) of the nomogram model for predicting the risk of postoperative NVG in DR was 0.962 [95% confidence interval (95%CI): 0.932-0.991], and the sensitivity and specificity were 95.2% and 89.2%, respectively, suggesting that the nomogram model had good differentiation ability (Figure 2A). After 500 repeated samples of the original data, a calibration curve was constructed (Figure 2B). The average absolute error of the calibration curve was 0.024, indicating that the degree of calibration and prediction consistency of the nomogram was high. In the Hosmer-Lemeshow goodness of fit test χ^2 = 2.854 (P = 0.943 > 0.05), the difference between the predicted risk and the observed risk was small; therefore, the NVG predicted by the model was in good agreement with the actual risk.

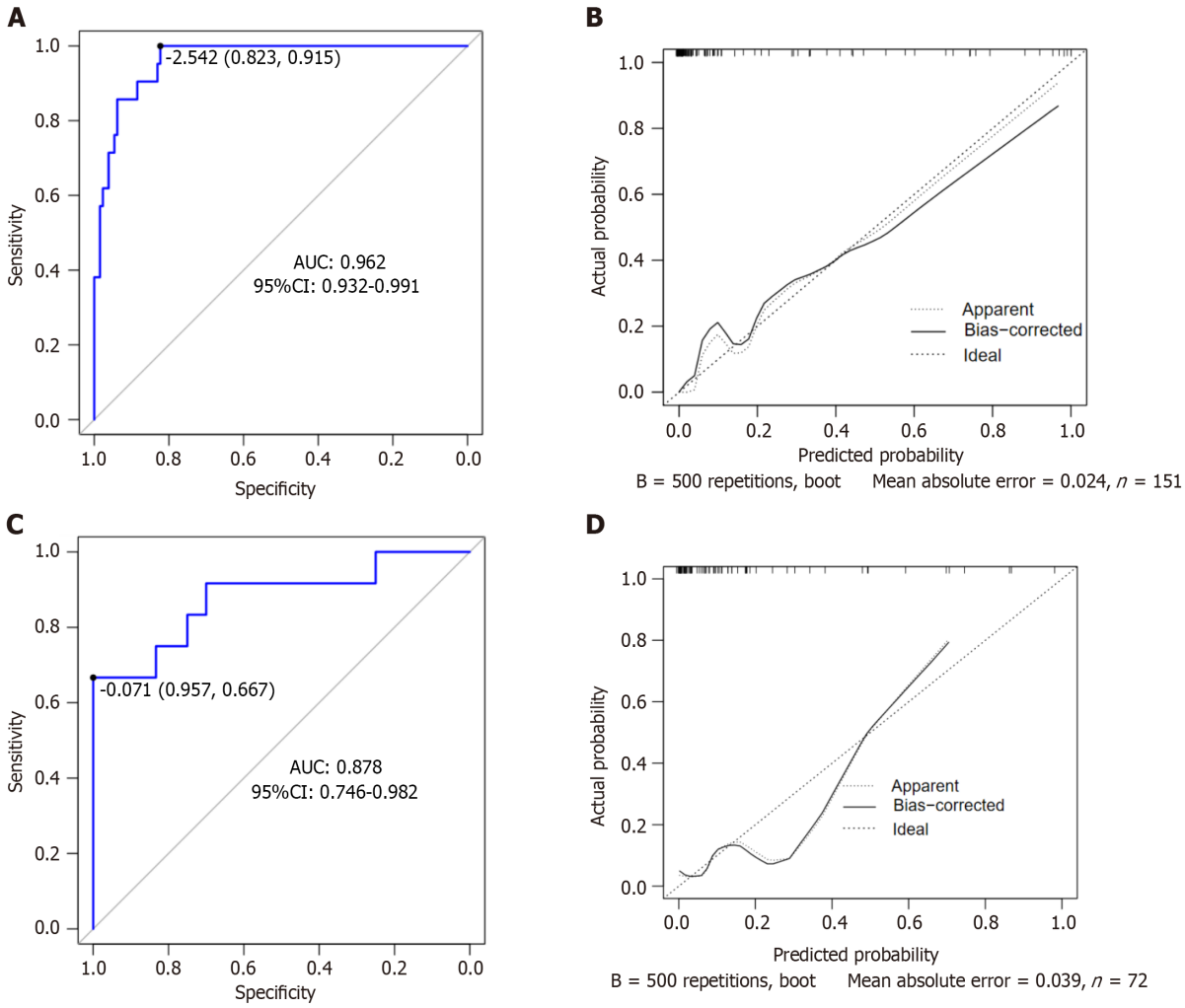

Figure 2 The prediction performance of the model was evaluated on the internal and external validation set. A: Receiver operating characteristic (ROC) curve of the internal validation set; B: Calibration curves of the internal validation set; C: ROC curve of the external validation set; D: Calibration curves for the external validation set. AUC: Area under the curve; CI: Confidence interval; ROC: Receiver operating characteristic.

External validation performance of the nomogram

The input validation set data for external verification showed that the AUC, sensitivity, and specificity were 0.878 (95%CI: 0.746-0.982), 66.7%, and 95.7%, respectively, indicating high prediction accuracy (Figure 2C). The correction curve was close to the ideal value (the average absolute error was 0.039), showing a prediction probability consistent with the measured value (Figure 2D).

DISCUSSION

NVG is a common and refractory complication in patients with DR. Previous studies have shown that young age, coronary heart disease or cerebral infarction, cataract phacoemulsification surgery, ipsilateral carotid artery stenosis, residual non-perfusion area of the retina after PPV, poor intraoperative retinal photocoagulation effect, postoperative retinal redetachment, multiple operations, and perioperative blood glucose instability are independent risk factors for postoperative NVG in patients with DR[11]. However, evidence on the relationship between serum and aqueous humor inflammatory factors and postoperative NVG in patients with DR is insufficient, and further research is needed.

NVG is caused by the release of various cytokines (such as VEGF) induced by retinal ischemia and hypoxia, thus promoting neovascularization, obstructing aqueous humor circulation, and increasing intraocular pressure[12]. The results of this study showed that the risk factors for postoperative NVG in patients with DR were posterior capsule defects during cataract surgery, preoperative VEGF ≥ 302.90 pg/mL, HbAlc ≥ 9.05%, aqueous fluid IL-6 ≥ 53.27 pg/mL, and aqueous fluid IL-10 ≥ 9.11 pg/mL. Posterior capsular defects during cataract surgery are correlated with postoperative NVG in patients with DR[13]. According to our results, the risk of postoperative NVG in posterior capsular defects during cataract surgery was 11.868 times that of posterior capsular integrity defects. The potential mechanism of NVG induced by posterior capsule defects during cataract surgery involves the release of several cytokines (such as VEGF) because of DR retinal ischemia and hypoxia. Simultaneously, the vitreous fluid fills up, and VEGF spreads faster,

resulting in a posterior capsule defect. VEGF enters the posterior chamber through the damaged barrier and circulates to the iris and horn of the atrium along with the aqueous solution to form new blood vessels, resulting in NVG[14]. Palfi Salavat *et al*[15] have shown that anti-VEGF drugs can effectively prevent the formation of posterior capsule blood vessels and that the postoperative use of anti-VEGF drugs can prevent the occurrence of NVG. In addition, Simha *et al*[16] showed that anti-VEGF drugs effectively reduced intraocular pressure in patients with NVG. Our study also found that VEGF is a risk factor for NVG formation and a sensitive factor for predicting postoperative NVG in patients with DR, which is consistent with the results of previous studies by other medical researchers. Excessive secretion of VEGF can promote the generation of new blood vessels at the iris surface and anterior chamber angle, which further suggests that VEGF may be involved in the mechanism of NVG secondary to DR. Hase *et al*[17] found high VEGF-C expression in the trabecular meshwork tissues of patients with glaucoma. In the case of severe retinal ischemia, VEGF expression promotes the formation of NVG[18]. HbA1c is the product of the combination of hemoglobin in red blood cells and serum sugars (mainly glucose) through a non-enzymatic reaction, which mainly reflects the changes in blood sugar in the previous 2 months. It can change the affinity of red blood cells to oxygen such that tissues and cells are deprived of oxygen. HbA1c is one of the biomarkers of DR, and the gradual accumulation of HbA1c concentration is closely related to the occurrence and progression of the disease[19]. Sakamoto *et al*[20] believe the HbA1c difference to be a risk factor for NVG occurrence. Tissue hypoxia caused by increased HbA1c content may lead to retinal hemorrhage, exudation, edema, ischemia, and eventually neovascularization.

Extensive and in-depth studies on NVG have found that its pathogenesis is not only related to angiogenesis caused by ischemia and hypoxia-induced increase in VEGF expression but also to inflammation[21]. Several studies[22,23] have found that inflammatory factor levels in serum and aqueous fluid are increased in patients with NVG and are positively correlated with VEGF levels, suggesting that inflammatory factors have angiogenesis-promoting activities. Our study also showed that high levels of IL-6 and IL-10 in the aqueous humor increased the risk of postoperative NVG in patients with DR. IL-6 is a major cytokine secreted by immune T cells and is involved in immune response and inflammation. Ocular IL-6 is produced by the retinal pigment epithelium, corneal epithelium, and other cells[24,25]. An abnormal increase in IL-6 expression in the aqueous humor indicates an active ocular inflammatory response. Polidoro *et al*[26] reported that IL-6 increased VEGF expression. IL-10 may promote ocular neovascularization by stimulating VEGF expression[27]. It also promotes ocular neovascularization by regulating macrophages during retinal hypoxia[27]. In this study, there was no significant correlation between the levels of inflammatory factors in the serum and aqueous humor. A possible reason is that although the ocular inflammatory response was active and the blood-eye barrier was damaged to some extent during the test period, its function was not destroyed, and the entry of some inflammatory factors into the blood was blocked; therefore, there was no consistency between the serum and aqueous humor cytokine levels.

Based on the individual risk indicators, this study established a nomogram model for predicting individual risk factors. The model expressed the contribution rate of each risk index based on the length of the line segment, which is intuitive, concise, readable, and practical in clinical practice. We used internal and external data to verify the accuracy of the model, finding the model to have a high degree of differentiation and the actual prediction curve matching the ideal. Clinically, the risk of postoperative NVG in patients with DR can be predicted according to the scores of each risk factor to strengthen the intervention of controllable factors. Clinical staff should attach great importance to patients with posterior capsular defects during combined cataract surgery or elevated concentrations of preoperative VEGF, HbAlc, aqueous humor IL-6 and aqueous humor IL-10, and actively adopt preventive measures. However, because the sample size of this study was limited to a single center, there is a potential selectivity bias. It was also difficult to obtain more clinical data, which may have resulted in missing potential risk factors. Therefore, the results of this study need to be verified by multi-center and large sample size research and mass data mining.

CONCLUSION

In summary, the primary influencing factors for NVG in patients with DR after surgery include posterior capsular defect, preoperative VEGF, HbAlc, aqueous fluid IL-6, and aqueous fluid IL-10. Furthermore, constructing a demographic model based on risk factors yields high prediction accuracy. This study can provide a reference for clinical personnel to screen high-risk groups and formulate relevant preventive and treatment measures.

ARTICLE HIGHLIGHTS

Research background

Pars plana vitrectomy (PPV) can effectively treat diabetic retinopathy (DR); however, some patients are prone to neovascular glaucoma (NVG) after surgery, affecting treatment efficacy. An in-depth understanding of the risk factors for NVG formation and the construction of an effective prediction model are important for clinical intervention to reduce the occurrence of NVG.

Research motivation

Previous studies on NVG risk factors did not include inflammatory factors in their analysis, and there is a lack of a quick and effective clinical risk prediction model. A thorough understanding of the risk factors for NVG and the construction of an effective risk assessment model can promote the clinical identification of high-risk patients and guide interventions.

Research objectives
To analyze the risk factors (including inflammatory factors) for NVG after PPV in patients with DR, build a nomogram model based on this, and evaluate the effectiveness of the model.

Research methods
Binary logistic regression was performed to analyze the risk factors for NVG in patients with DR after PPV. The R language (R 4.0.3) software was used to construct the nomogram, and its accuracy and effectiveness were evaluated using receiver operating characteristic (ROC) and calibration curves.

Research results
Risk factors for NVG after PPV in DR include posterior capsule defect during combined cataract surgery, preoperative VEGF, HbAlc, aqueous fluid IL-6, and aqueous fluid IL-10, and the column nomogram model constructed based on this had good differentiation [AUC: 0.962 (95% confidence interval, 95%CI): 0.932-0.991), sensitivity: 91.5%, and specificity: 82.3%]. The external validation of the model was also good [AUC: 0.878 (95%CI: 0.746-0.982), sensitivity: 66.7%, specificity: 95.7%].

Research conclusions
NVG influencing factors in patients with DR after surgery are related to many factors, including posterior capsular defects, preoperative VEGF, HbAlc, aqueous fluid IL-6, and aqueous fluid IL-10. The nomogram built based on risk factors had a high prediction accuracy and clinical applicability and is expected to expand the scope of application and reduce the occurrence of NVG.

Research perspectives
This study confirmed that the constructed column diagram is suitable for DR after PPV at our hospital. Future research should aim to expand the sample size to multiple centers to enhance the reliability of the results and facilitate the popularization and application of the model.

FOOTNOTES

Co-first authors: Yi Shi and Yan-Xin Zhang.

Co-corresponding authors: Ai-Hua Liu and Xiao-Rong Li.

Author contributions: Shi Y and Zhang YX designed and performed the research and wrote the paper; Liu AH and Li XR designed the research and supervised the report; Jiao MF, Ren XJ, and Hu BJ contributed to the analysis; all authors were involved in the critical review of the results and have contributed to, read, and approved the final manuscript. Shi Y and Zhang YX contributed equally to this work and are co-first authors; Liu AH and Li XR contributed equally to this work and are co- corresponding authors. The reasons for designating Liu AH and Li XR as co-corresponding 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, Liu AH and Li XR contributed efforts of equal substance throughout the research process. The choice of these researchers as co-corresponding authors acknowledges and respects this equal contribution, while recognizing the spirit of teamwork and collaboration of this study.

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ORIGINAL ARTICLE

Clinical Trials Study

Effect of special types of bread with select herbal components on postprandial glucose levels in diabetic patients

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Abstract

BACKGROUND

Nutrition recommendations in patients with type 2 diabetes mellitus (T2DM) are to consume rye or integral bread instead of white bread. A positive effect on glucoregulation has been achieved by enriching food with various biologically active substances of herbal origin, so we formulated an herbal mixture that can be used as a supplement for a special type of bread (STB) to achieve better effects on postprandial glucose and insulin levels in patients with T2DM.

AIM

To compare organoleptic characteristics and effects of two types of bread on postprandial glucose and insulin levels in T2DM patients.

METHODS

This trial included 97 patients with T2DM. A parallel group of 16 healthy subjects was also investigated. All participants were given 50 g of rye bread and the same amount of a STB with an herbal mixture on 2 consecutive days. Postprandial blood glucose and insulin levels were compared at the 30th, 60th, 90th and 120th min. A questionnaire was used for subjective estimation of the organoleptic and satiety features of the two types of bread.

RESULTS
Compared to patients who consumed rye bread, significantly lower postprandial blood glucose and insulin concentrations were found in T2DM patients who consumed STB. No relevant differences were found among the healthy subjects. Subjectively estimated organoleptic and satiety characteristics are better for STB than for rye bread.

CONCLUSION
STB have better effects than rye bread on postprandial glucoregulation in T2DM patients. Subjectively estimated organoleptic and satiety characteristics are better for STB than for rye bread. Therefore, STB can be recommended for nutrition in T2DM patients.

Key Words: Special types of bread; Postprandial glucoregulation; Insulin; Nutrition; Type 2 diabetes mellitus

Core Tip: We are testing special types of bread in populations with compromised glucoregulation. It is novel, tasty, and very effective in postprandial glucoregulation. In this study, we compared novel bread (TopiGluk) with standard hospital rye bread and obtained significant differences regarding glucose metabolism of TopiGluk compared to rye bread. This research might be interesting for readers because TopiGluk could become a standard supplement in bread for patients with compromised glucoregulation and those at high risk for diabetes.

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INTRODUCTION

Diabetes mellitus (DM) is one of the main health issues worldwide, and its incidence has been increasing for several decades[1,2]. Additionally, many people have reduced glucose tolerance[3]. A Global Report on Diabetes showed that the number of adult DM patients increased almost fourfold between 1980 and 2014, going from 108 million patients to 422 million patients[4]. This dramatic increase is mostly due to type 2 DM (T2DM) and its risk factors, especially obesity[5].

Dietary interventions are the main economic and effective strategies aimed at reducing blood glucose and insulin levels in the population[3,6]. Physical activity also improves glucose tolerance as a supplemental treatment for obesity but not as a replacement for dietary measures[7]. Controlled nutrition improves glucose tolerance by reducing endogenous glucose production and improving sensitivity to insulin[8]. The consumption of foods with a low glycemic index (GI) decreases postprandial blood glucose and insulin levels and their fluctuations[9,10].

One of the basic foodstuffs worldwide is bread. Recommendations for this population include the consumption of rye or whole grain bread with higher dietary fiber (DF) content instead of white bread[11]. Several trials have shown that a positive effect on glucoregulation may also be achieved by enriching food with various biologically active substances of herbal origin[12].

TopiGluk bread was patented by the National Intellectual Property Office (registration No. 73932) as a supplementary treatment for people with disease. TopiGluk bread is made by adding a mixture of nutrients to whole wheat, oat and buckwheat flour.

We hypothesized that TopiGluk bread would significantly improve glucose tolerance in T2DM patients. The aim of this study was to evaluate the effects of TopiGluk bread on postprandial glucose levels in T2DM patients. Additionally, the insulin levels and organoleptic properties of the examined bread were evaluated as secondary objectives.

MATERIALS AND METHODS

Time and place of study

A prospective study was conducted at the Clinic of Endocrinology, Diabetes and Metabolic Disorders, Clinical Center of Serbia, Belgrade. The study was carried out from 20 May 2016 to 25 March 2017.

Subjects

The trial included 97 patients with T2DM who were treated as outpatients at the Clinic of Endocrinology, Diabetes and Metabolic Disorders, Faculty of Medicine, University of Belgrade. The inclusion criteria were 18 years or older and

confirmed T2DM. The exclusion criteria were acute T2DM complications; acute inflammatory conditions; chronic diseases of the liver or gastrointestinal tract; malignant diseases; immunodeficiency; narcotic or alcoholic addictions; and pregnancy (Figure 1).

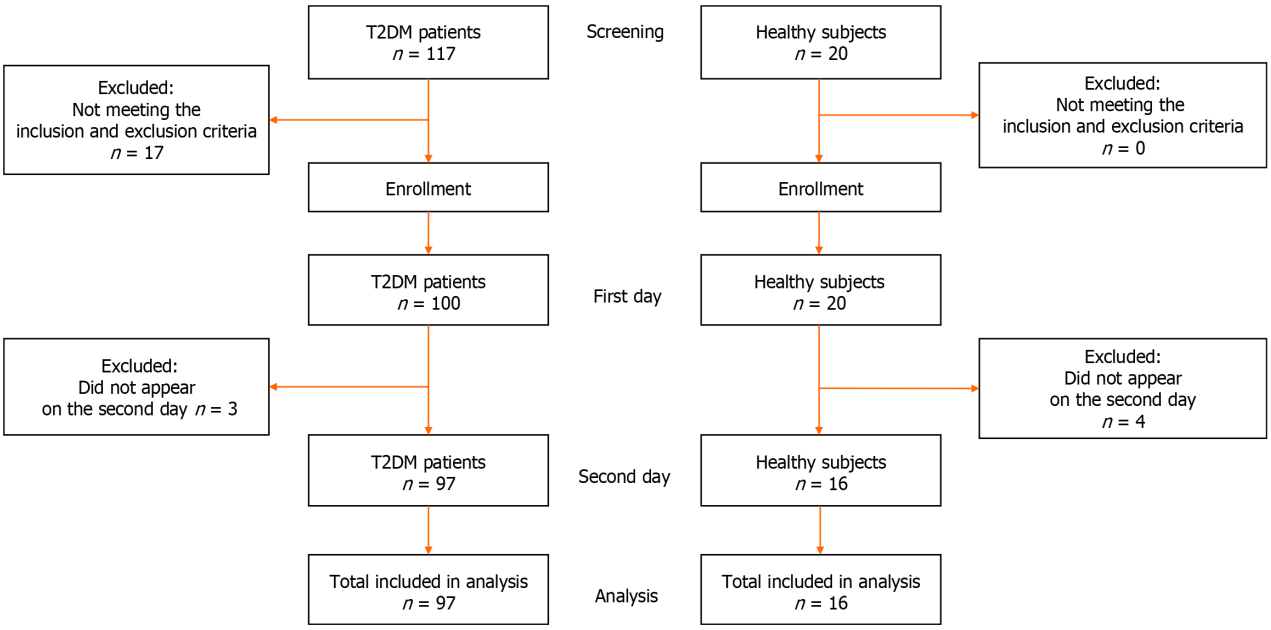

Figure 1 CONSORT flowchart. T2DM: Type 2 diabetes mellitus.

A parallel group of 16 healthy controls were analyzed; they had no history of metabolic disorders (including DM) and had preserved glucose homeostasis based on their medical history.

All of the subjects provided informed consent for inclusion in the study. The investigation was carried out in accordance with the Declaration of Helsinki and was revised in 2013. The study was carried out in accordance with the EU Directive 20/2001/EC, the Commission Directive 2005/28/EC, and the European Parliament Declaration on Good Clinical Practice, as well as the International Conference on Harmonization ICH-GCP (E6); approval for this research was given by the Ethics Committee, Clinical Center of Serbia, No. 105/39 on 19 May 2016. The study is registered in the German Clinical Trials Register DRKS00023611. The clinical trial was registered after completion because the law of the Republic of Serbia does not require registration in the international registry. The authors confirm that all ongoing and related trials for this food supplement have been registered.

Study protocol

Prior to the trial, a detailed history was taken, which included data on physical activity and lifestyle, the duration of T2DM and therapy, chronic complications of T2DM and nutritional habits. Data on anthropometric characteristics, weight and height were obtained from medical records if no significant changes were observed in the last 6 mo. Body mass index was calculated by dividing the body weight in kilograms by the squared body height in meters (kg/m^2). All participants were instructed to fast for at least 12 h prior to blood sampling.

A slice of bread weighing 50 g was provided to each participant, which is the usual portion for T2DM patients. The nutritional content of the bread is presented in Table 1. One day, all participants were given a piece of rye bread, and on another day, they were given a piece of special bread. Patients were advised to perform similar activities on both days. Furthermore, patients were required to fast for 12 h prior to each blood sampling.

Participants were instructed to complete a questionnaire about the look, smell, taste, quantity, and satiety of the bread. Answers were given on a numerical scale from 1 (worst) to 5 (best). Upon arrival at the clinic at approximately 08:00 in the morning, the responsible researcher accommodated each participant in the laboratory. A venous cannula was inserted in the arm of the patient. The first blood sample was taken before the meal. Next, four blood samples were taken every 30 min after the meal. Healthy subjects were with evaluated with a glucose meter, and blood samples were collected using finger sticks (capillary blood sampling) in the infirmary.

The laboratory analyses of T2DM patients included hemoglobin A1c (HbA1c) and C-peptide levels (at baseline only) and glucose and insulin levels (at baseline and 30 min, 60 min, 90 min and 120 min after the meal) on both examination days. Blood glucose measurements were performed using the spectrophotometric (hexokinase) method with a COBAS 6000 (Roche Diagnostics, Basil, Switzerland) with a reference range of 3.9-6.1 mmol/L. Insulin and C-peptide levels were measured using an immunoradiometric assay method with a gamma counter for *in vitro* diagnosis (LKB-WALLAC ChinGamma Model 1272).

Laboratory analysis of healthy controls included only glucose levels at baseline (before the meal) and at 30 min, 60 min, 90 min and 120 min after the meal. Blood measurements from capillary blood were performed with an electrochemical

Table 1 Comparative nutritional composition of the rye bread and TopiGluk bread (100 g)

Name of nutrient (unit)	Rye bread	TopiGluk bread	RDI, %
Energy (kJ)	1072	1088	12.95
Energy (kcal)	253	259	12.95
Fat (g)	1.6	6.6	9.43
Saturated fatty acids (g)	0.6	1.2	6
Carbohydrates (g)	48.6	37.5	14.42
Sugars (g)	0.1	3.7	4.11
Dietary fibers (g)	3.8	8.6	143.3
Proteins (g)	9.2	8.1	16.2
Salt (g)	1.4	1.1	18.3
Magnesium (mg)	-	72.6	
Zinc (mg)	-	1.6	
Chromium (μg)	-	20.1	
Selenium (μg)	-	5.3	

RDI: Reference daily intake.

method using a Biosen C-Line machine (EKF Diagnostics, Cardiff, United Kingdom) with a reference range of 3.9-6.1 mmol/L.

Due to differences in sampling methods, diabetic and healthy subjects were not compared, except for blood sugar changes from baseline to 120 min (the sampling method did not affect the change).

Differences between 30 min and baseline, 60 min and baseline, 90 min and baseline and 120 min and baseline levels were calculated (delta glucose and delta insulin). The area under the curve (AUC) was calculated using a trapezoidal model[13].

Ingredients of special type of bread

The bread was a brand of the Delhaize Serbia distributor in cooperation with partners for product development: The international company Puratos and the national bakery products manufacturer Alimpije-and with a partner for product improvement-ZZ Zdravlje (Čačak, Serbia), the inventor of TopiGluk®.

The ingredients of TopiGluk® bread are as follows: Basil (*Ocimum basilicum*), garlic (*Allium sativum*), Greek seed (*Trigonella foenum graecum*), ginger (*Zingiber officinale*), oat (*Avena sativa*), Jerusalem artichoke (*Helianthus tuberosus*), and cinnamon (*Cinnamomum verum*). Sunflower and linen seeds are also among the supplements. The nutritional content of 100 g of the special type of bread (STB) with the TopiGluk mixture is presented in Table 1. Approximately one-third of the mentioned mixture contains active principles (TopiGluk), while the remaining two-thirds are equivalent parts buckwheat, oat and whole wheat flour. The representation of the components in TopiGluk was 0.5% garlic, 0.5% ginger, 2% basil, 3% Greek seed, 5% cinnamon, 8% oat, and 13% Jerusalem artichoke.

Statistical analysis

A sample size of 97 T2DM patients with achieved 90% power to detect a mean difference of 1.0 ± 3.0 mmol/L with a significance level for alpha error of 0.05 using a two-sided paired samples *t* test.

The results are presented as counts (%) or means ± SD depending on the data type and distribution. Measurements were compared using parametric (paired sample *t* test) and nonparametric (Wilcoxon signed rank test) tests. Glucose changes (delta) between healthy subjects and patients were compared using an independent samples *t* test. Deltas were calculated as the difference between the 30 min, 60 min, 90 min and 120 min time points and baseline. The AUC was calculated using the trapezoidal rule. All *P* values less than 0.05 were considered significant. All the data were analyzed using R 3.4.2. R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (https://www.R-project.org/).

RESULTS

The majority of participants were males in the 6th to 7th decade of life, preobese to obese individuals, and physically active individuals. The majority of patients were receiving oral antidiabetic therapy, while one-third were receiving insulin. Coronary disease and neuropathy were present in one-third of patients. The average duration of diabetes was shorter than 10 years. Most of the patients consumed rye bread, for which the median daily intake was 1 slice, while other bread

types (whole wheat bread and white bread) were less represented in terms of nutritional status (both medians were 0) (Table 2).

All the evaluated characteristics were greater for the STB than for rye bread, as presented in Table 3.

In T2DM patients, we found better glucoregulation after the STB portion than after the rye bread portion. Significantly lower delta values of blood glucose were observed after the STB portion at the 90th and 120th min than after the rye bread portion. In healthy subjects, no significant differences in blood glucose delta values were observed, except at the 90th min. Concerning the AUC of blood glucose, a significant difference was found only at the 90th min. In T2DM patients, the AUC was 21.2% ± 5.1% after the rye bread portion and 19.6% ± 4.5% after the STB with TopiGluk portion ($P < 0.001$). In healthy subjects, no significant differences in the AUC were found after the two types of bread were consumed ($P = 0.924$) (Figure 2).

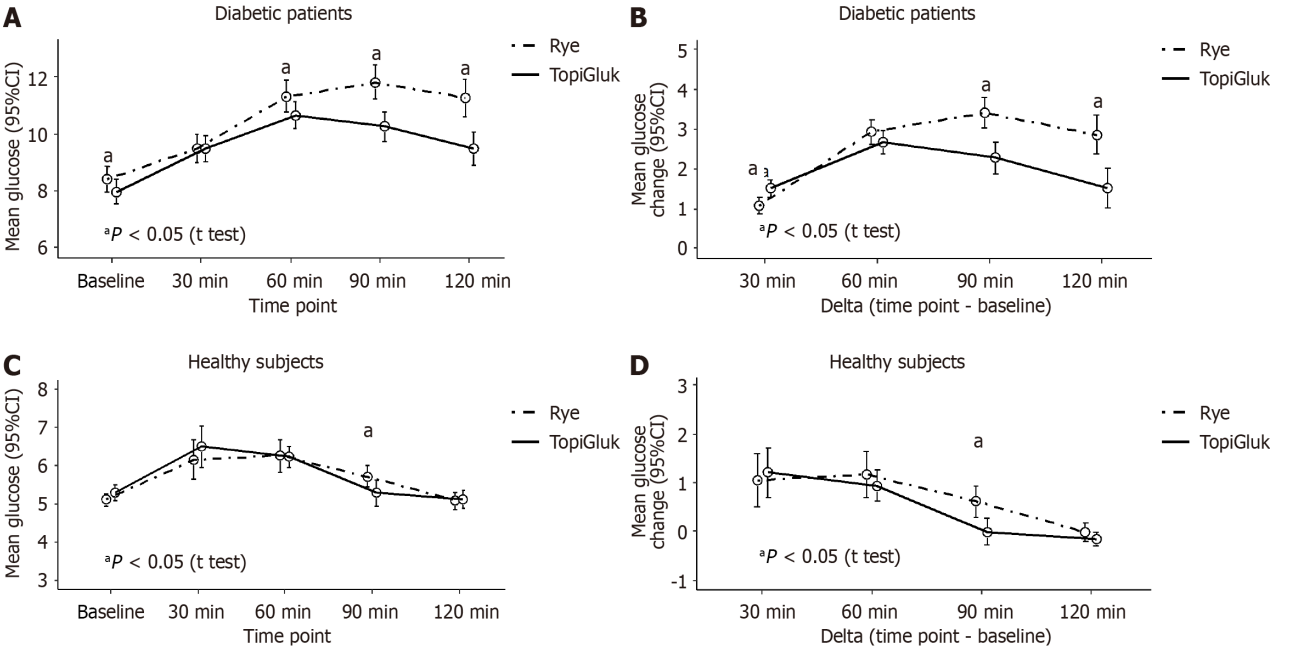

Figure 2 Mean glucose levels and mean glucose change (in mmol/L) after the consumption of rye bread and the special type of bread with TopiGluk® in diabetes mellitus type 2 patients and healthy subjects. A: Absolute values of glucose in diabetic patients; B: Glucose change from baseline in diabetic patients; C: Absolute values of glucose in healthy subjects; D: Glucose change from baseline in healthy subjects. $^{a}P < 0.05$.

When comparing patients and healthy subjects regarding delta values, all differences were significant at the 0.001 level for both the rye bread and the STB with the TopiGluk® mixture, except for the change from baseline to 30 minutes after the rye ($P = 0.919$) and STB with TopiGluk® ($P = 0.313$) portions (Figure 2).

The insulin levels of the patients were analyzed at each time point (0, 30, 60, 90 and 120 min) and for each type of bread (Figure 3). Initially, the median insulin concentrations were identical. However, significant differences in insulin levels were observed at the 90th min and the 120th min after the two types of bread were consumed. After the STB with TopiGluk® was consumed, we found significantly lower median insulin concentrations compared to after rye bread was consumed. A comparison of the deltas revealed significant differences at the same time points. The median value and interquartile range of the AUC of insulin in T2DM patients with was 46.7 (29.7-61.1) after consuming rye bread and 39.9 (30.1-57.0) after consuming the STB with TopiGluk® ($P = 0.035$).

Diabetic patients were divided into two subgroups: patients receiving oral antidiabetic therapy and patients receiving insulin therapy (Figure 4). In patients on oral antidiabetic therapy, significantly higher blood glucose levels were observed at the 60th, 90th and 120th min after the rye bread was consumed than after the STB with TopiGluk® was consumed. Differences in blood glucose changes from baseline were significant at the 90th min and at the 120th min. In patients receiving insulin therapy, all mean glucose levels were significantly greater after rye bread was consumed than after the STB with TopiGluk® was consumed, but the changes (deltas) were significantly different only at the 90th min and at the 120th min (Figure 4).

DISCUSSION

This study compared the effects of rye bread and (STB) with TopiGluk® on glucoregulation. Patients subjectively assessed the STB as better than the rye bread regarding appearance, aroma, taste, amount, satiety level and duration of satiety. We observed lower glycemic and insulin levels from baseline to the 120th min in diabetic patients after the STB was consumed than after the rye bread was consumed. In healthy subjects, this difference was not observed, as expected.

Table 2 Basic characteristics of patients with type 2 diabetes mellitus

Characteristic	Value
Age in yr	61.1 ± 9.3
Sex as male	62 (62.9)
BMI in kg/m^2	29.2 ± 4.7
Physically active	83 (85.6)
Sedentary job	60 (61.94)
Oral antidiabetic therapy	86 (88.7)
Insulin therapy	28 (28.8)
Combined	15 (50.0)
Conventional	4 (13.3)
Intensive	11 (36.7)
Duration of DM in yr	7 (3-12)
HbA1c	7.6 ± 1.5
C peptide in ng/mL	1.23 ± 0.78
Complications of DM	
Coronary disease	34 (35.1)
Cerebrovascular disease	7 (7.2)
Peripheral vascular disease	21 (21.6)
Retinopathy	25 (25.87)
Neuropathy	35 (36.1)
Nephropathy	12 (12.4)
Diabetic foot	4 (4.1)
Main bread in nutrition	
Rye	52 (53.6)
Whole wheat	40 (41.2)
White wheat	35 (36.1)

Data are mean ± SD or *n* (%). BMI: Body mass index; DM: Diabetes mellitus; HbA1c: Hemoglobin A1c.

The STB with TopiGluk® can be described as a "functional food" because it may have the following positive effects on glucose metabolism: decreased glucose absorption from the intestine to blood, increased glucose utilization, increased cell sensitivity to insulin, reduced insulin resistance, increased endogenous insulin production and increased glycogen in the liver[14].

Glucose absorption has been evaluated in several studies. Glucose absorption can be reduced using foods rich in DFs. DFs include lignin and a range of polysaccharides derived from cell walls that are poorly digested in the upper intestine [15]. DM patients are usually instructed to eat rye bread, which is rich in DF. Viscous and gel-forming soluble DFs inhibit macronutrient absorption and reduce the postprandial glucose response. However, in prospective cohort studies, insoluble cereal DF and whole grains, but not soluble DF, are consistently associated with reduced DM risk, suggesting that further unknown mechanisms are likely involved[16,17]. TopiGluk® contains whole wheat flour, buckwheat and oatmeal, which are rich in DF, especially beta glucans. TopiGluk is also rich in the soluble DF inulin from Jerusalem artichoke, which binds water and forms a viscous solution that delays gastric emptying and intestinal transit, thus reducing glucose absorption. This leads to a decreased blood glucose response[16,17]. DF also decreases insulin secretion and reduces the chance of reactive hypoglycemia during the postprandial period. On the other hand, this promotes satiety and satiation, increases fat oxidation and decreases fat storage[18].

Several authors have examined the effect of whole-meal and whole-kernel rye breads on glucose metabolism compared to that of white wheat bread. Leinonen *et al*[19] concluded that whole kernel rye bread has no effect on the glucose response but has an effect on the postprandial insulin response. In healthy subjects, we found no differences in blood glucose levels after consumption of the STB with TopiGluk® or after consumption of rye bread. This is probably due to preserved regulatory mechanisms of glucose metabolism in healthy subjects.

Table 3 Subjective assessment of the quality of bread/scale from 1 to 5

Characteristic	Bread		*P* value[1]
	Rye	TopiGluk	
Appearance	3.42 ± 1.02	4.25 ± 1.01	< 0.001
Aroma	3.33 ± 1.08	4.28 ± 1.06	< 0.001
Taste	3.37 ± 1.05	4.34 ± 1.06	< 0.001
Satisfaction with the amount	4.10 ± 1.16	4.33 ± 1.05	0.057
Satiety level	3.91 ± 1.04	4.45 ± 0.84	< 0.001
Duration of satiety	3.67 ± 1.12	4.43 ± 0.84	< 0.001

[1]*t* test.
Data are mean ± SD.

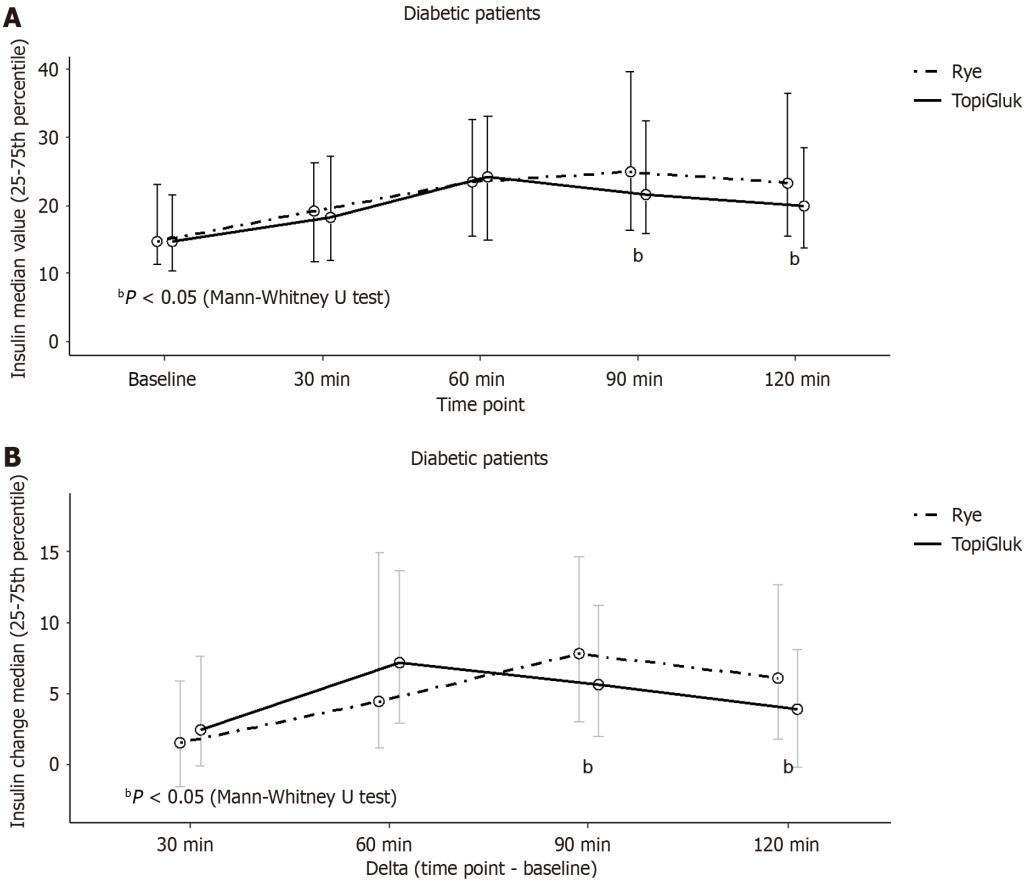

Figure 3 Insulin levels (μIU/L), median (25-75th percentile) and change after the consumption of rye bread and the special type of bread with TopiGluk® in diabetic patients. A: Absolute values; B: Change compared to baseline. $^{b}P < 0.05$.

However, in T2DM patients, we found lower blood glucose and insulin levels after the consumption of the STB with TopiGluk® than after the consumption of the rye bread. This approach is very important for individuals with impaired glucose tolerance or an increased risk of diabetes. Starchin bread decomposes during digestion to simple sugars, which affect glucoregulation. TopiGluk is a mixture of natural metabolically active ingredients that play a proven role in metabolic regulation. Studies in diabetic rats revealed the significant effect of basil on glucose metabolism and its potential usefulness in treating T2DM patients[20]. This effect is likely mediated by decreased glucose absorption and glucose mobilization in the liver[20]. A recent meta-analysis revealed that garlic, in addition to therapy, contributes to improved glucose and lipid control[21,22]. Fenugreek is mostly produced in Eastern countries, but in the West, it is often used as a medicinal herb or spice. A recent meta-analysis revealed that fenugreek acutely reduces postprandial glucose levels, but the long-term effect on glucose metabolism has not been sufficiently tested[23]. A meta-analysis by Huang *et al*[24] revealed that ginger has no significant effect on fasting glucose levels but significantly improves HbA1c levels. Another meta-analysis by Hou *et al*[25] revealed the significant effect of oat on fasting blood glucose, HbA1c and lipid levels. Oat

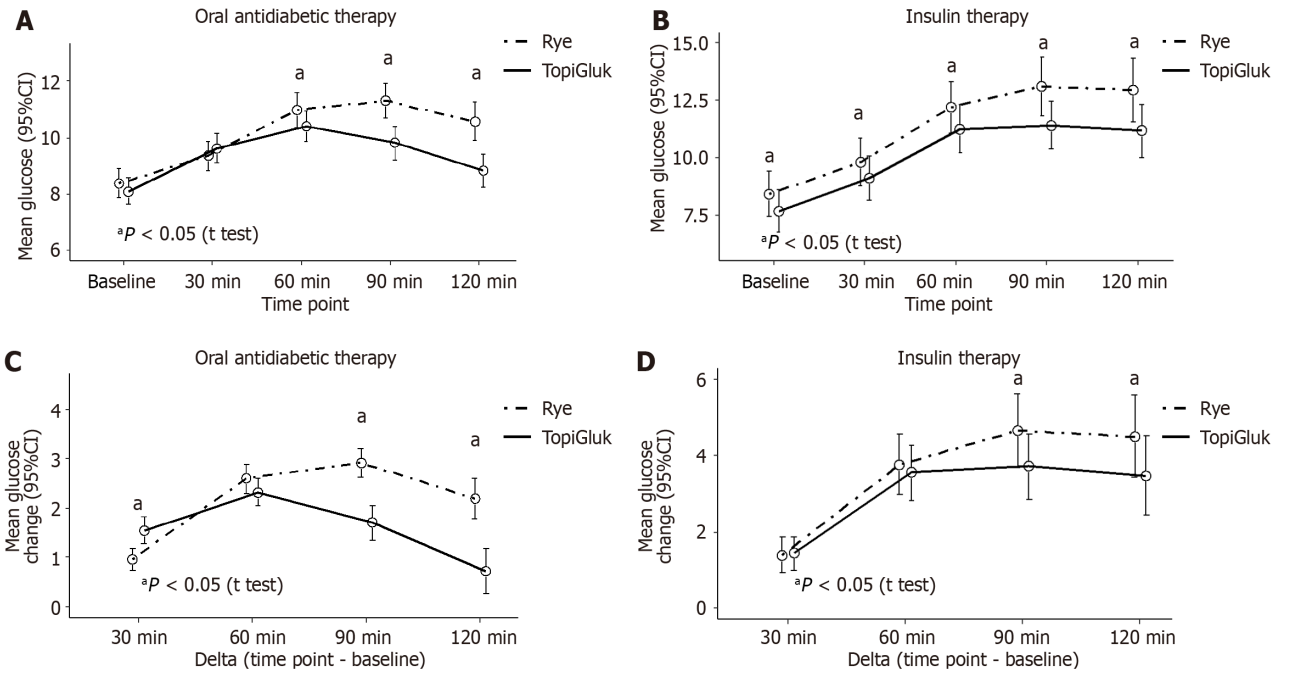

Figure 4 Insulin levels (μIU/L), median (25-75th percentile) and change after the consumption of rye bread and the special type of bread with TopiGluk® in diabetic patients. A: Absolute values in patients with oral antidiabetic therapy; B: Change compared to baseline in patients with insulin therapy; C: Change compared to baseline in oral antidiabetic therapy patients; D: Change compared to baseline in insulin therapy patients. $^{a}P < 0.05$.

is a common choice for people with obesity, digestive problems or diabetes. It is a grain with a low GI and is rich in vitamins and minerals. Jerusalem artichoke is a sunflower plant that favorably affects blood sugar levels. Its most valuable component is inulin. Dry-ground Jerusalem artichoke contains 70% inulin. Fructose is a result of inulin hydrolysis and is an excellent replacement for glucose. A study of diabetic rats revealed that Jerusalem artichoke improved metabolism, microcirculation, and blood vessel conditions and thereby reduced the severity of diabetes complications[26]. A recent study of newly diagnosed DM patients revealed a positive effect of Jerusalem artichoke on postprandial glucose levels[27]. Cinnamon, a spice with high global consumption, also affects fasting blood glucose levels in T2DM and prediabetic patients[12,28,29]. While choosing biologically active substances for TopiGluk®, numerous factors should be considered; the most important are the availability of the substance, its impact on glucoregulation, other favorable metabolic effects, its applicability in the technological process of bread preparation and manufacturing, and the impact of certain phases of the technological procedure; for example, the impact of the bread baking temperature on the active principles of the ingredients (duration), specific aroma and taste at planned concentrations, mutual compatibility and cumulative effect on glucoregulation.

A comparison of the organoleptic properties of two kinds of bread, an STB with TopiGluk® and rye bread, revealed that the former was better than the latter. A number of respondents emphasized the sweet taste of the STB with TopiGluk®, which may be particularly important for DM patients because they are usually not allowed to consume sweet food.

The results of our research are consistent with the latest data from the literature indicating that the plants added to the TopiGluk® bread (*Ocimum basilicum*, *Allium sativum*, *Trigonella foenum graecum*, *Zingiber officinale*, *Avena sativa*, *Helianthus tuberosus*, and *Cinnamomum verum*) have antidiabetic properties[30-35].

CONCLUSION

Based on the present results, we can conclude that postprandial blood glucose levels in T2DM patients are lower after consuming TopiGluk bread than after consuming the same amount of rye bread. Improved glucoregulation was noted in T2DM patients at 90 and 120 min, both in patients who were taking oral antidiabetic drugs and in patients receiving insulin therapy. An STB made with TopiGluk® has better subjectively assessed organoleptic and fine characteristics than rye bread. The STB with TopiGluk® can be recommended as part of the diet in T2DM patients.

ARTICLE HIGHLIGHTS

Research background

Bread that we are testing is novel, tasty and very effective in populations with compromised glycoregulation. In our study, we compared novel bread with standard hospital rye bread and obtained significant differences regarding glucose

metabolism of special type of bread (STB) with TopiGluk compared to rye bread.

Research motivation

All participants were given 50 g of rye bread or STB with herbal mixture on 2 consecutive days. In the continuation of these studies, it would be interesting to increase the amount of tested bread and see how it would affect postprandial glycemia.

Research objectives

To compare organoleptic characteristics of two sorts of bread and their effects on postprandial glucose and insulin levels in type 2 diabetes mellitus (T2DM) patients.

Research methods

Postprandial blood glucose and insulin levels were examined on 2 consecutive days after the consumption of rye bread and a special type of bread with an herbal mixture. A questionnaire was used for comparison of the organoleptic properties of two kinds of bread.

Research results

A special type of bread with an herbal mixture caused significantly lower postprandial blood glucose in T2DM patients than rye bread, and it showed better organoleptic and satiety characteristics.

Research conclusions

Our study showed a significant difference in postprandial blood glucose and insulin levels between patients that consumed rye bread and those that consumed a special type of bread with herbal mixture. This special type of bread has better effects on postprandial glucoregulation in T2DM patients.

Research perspectives

The results of this research can be the basis and incentive for future research that would determine which biochemical substances from plant components added to STB are responsible for the effect of postprandial glycemia.

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FOOTNOTES

Author contributions: Gostiljac DM, Popovic SS and Dimitrijevic-Sreckovic V designed the research study; Gostiljac DM, Ilic SM and Jevtovic JA performed the research; Gostiljac DM, Soldatovic IA and Nikolic DM analyzed the data and wrote the manuscript; All authors have read and approve the final manuscript.

Institutional review board statement: All subjects provided informed consent for inclusion in the study. The investigation was carried out in accordance with the Declaration of Helsinki and was revised in 2013.

Clinical trial registration statement: The study is registered in the German Clinical Trials Register DRKS00023611. The clinical trial was registered after completion because the law of the Republic of Serbia does not require registration in the international registry.

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

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ORIGINAL ARTICLE

Observational Study

Examining the association between delay discounting, delay aversion and physical activity in Chinese adults with type-2 diabetes mellitus

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Abstract

BACKGROUND

The role of physical activity in diabetes is critical, influencing this disease's development, man-agement, and overall outcomes. In China, 22.3% of adults do not meet the minimum level of physical activity recommended by the World Health Organization. Therefore, it is imperative to identify the factors that contributing to lack of physical activity must be identified.

AIM

To investigate the relationship among delay discounting, delay aversion, glycated hemoglobin (HbA1c), and various levels of physical activity in Chinese adults diagnosed with type 2 diabetes mellitus (T2DM).

METHODS
In 2023, 400 adults with T2DM were recruited from the People's Hospital of Linxia Hui Autonomous Prefecture of Gansu Province. A face-to-face questionnaire was used to gather demographic data and details on physical activity, delay discounting, and delay aversion. In addition, HbA1c levels were measured in all 400 participants. The primary independent variables considered were delay discounting and delay aversion. The outcome variables included HbA1c levels and different intensity levels of physical activity, including walking, moderate physical activity, and vigorous physical activity. Multiple linear regression models were utilized to assess the relationship between delay discounting, delay aversion, and HbA1c levels, along with the intensity of different physical activity measured in met-hours per week.

RESULTS
After controlling for the sample characteristics, delay discounting was negatively associated with moderate physical activity (β = -2.386, 95%CI: -4.370 to -0.401). Meanwhile, delay aversion was negatively associated with the level of moderate physical activity (β = -3.527, 95% CI: -5.578 to -1.476) in the multiple linear regression model, with statistically significant differences.

CONCLUSION
Elevated delay discounting and increased delay aversion correlated with reduced levels of moderate physical activity. Result suggests that delay discounting and aversion may influence engagement in moderate physical activity. This study recommends that health administration and government consider delay discounting and delay aversion when formulating behavioral intervention strategies and treatment guidelines involving physical activity for patients with T2DM, which may increase participation in physical activity. This study contributes a novel perspective to the research on physical activity in adults with T2DM by examining the significance of future health considerations and the role of emotional responses to delays.

Key Words: Type 2 diabetes mellitus; Delay discounting; Delay aversion; Physical activity

Core Tip: The role of physical activity in the context of diabetes is paramount, influencing its development, management, and overall outcome. 22.3% of adults in China did not attain the minimum recommended level of physical activity outlined by the World Health Organization in 2018. Research has indicated that individuals' inability to engage in and maintain regular physical activity is partly attributable to a psychological inclination favoring immediate rewards over delayed, more substantial ones. Delay discounting, a concept rooted in behavioral economics. No investigations have been conducted on the correlation between delay discounting, delay aversion, and health-related aspects, such as physical activity, especially among Chinese adults diagnosed with type 2 diabetes mellitus. In this study, we found that elevated delay discounting and increased delay aversion correlated with reduced levels of moderate physical activity.

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INTRODUCTION

Diabetes mellitus constitutes a pervasive global public health concern. As of 2021, approximately 537 million individuals aged 20-79 years were afflicted with diabetes worldwide, with China having the largest burden, housing 140.9 million individuals. Projections indicate that by 2045, this figure is expected to escalate to 174.4 million[1]. Apart from leading to complications such as cardiovascular disease, retinopathy, neuropathy, and nephropathy, it is also associated with a mortality rate nearly twice that of individuals without diabetes[2]. In 2021, roughly 6.7 million adults aged 20-79 years succumbed to diabetes and its associated complications globally, accounting for 12.2% of all deaths within this age group worldwide, with China accounting for approximately 1.4 million of these deaths[1]. The global expenditure on health related to diabetes among adults aged 20-79 years has shown a rapid surge, escalating from 232 billion US dollars (USD) in 2007 to 966 billion USD in 2021. According to projections by the International Diabetes Federation, medical expenses associated with diabetes will reach 1.03 trillion USD in 2030 and 1.05 trillion USD by 2045. Notably, China's expenditures related to diabetes for adults aged 20 to 79 reached 165.3 billion USD in 2021, as the second position globally[1].

Physical inactivity is the fourth leading cause of mortality worldwide and is a modifiable risk factor[3,4]. Engaging in regular physical activity is a pivotal health behavior that mitigates and prevents the severity of numerous chronic ailments[5]. The role of physical activity in the context of diabetes is paramount, influencing its development,

management, and overall outcome[6]. Physical activity not only proves effective in managing blood glucose levels and reducing risk factors for cardiovascular disease in individuals with type 2 diabetes mellitus (T2DM)[7,8] but also serves as a preventive measure against or delay in the onset of diabetes-related complications[9]. It is noteworthy that consistent physical activity contributes to the reduction of glycated hemoglobin (HbA1c) levels, triglycerides, and blood pressure in individuals with T2DM[10], enhances insulin sensitivity[11], and diminishes the overall mortality risk for T2DM patients engaging in moderate to vigorous physical activity[12,13]. Additionally, physical activity plays a pivotal role in diminishing the risk of anxiety and depression[14,15], and psychological factors of particular significance in managing diabetes mellitus[16]. The World Health Organization (WHO) guidelines for physical activity and sedentary behavior recommend that adults partake in at least 150-300 minutes of moderate-intensity or 75-150 minutes of vigorous-intensity aerobic physical activity each week, or a combination thereof[17]. In 2016, 27.5% of adults globally failed to meet the WHO recommended standards for physical activity[18]. The results of China's chronic disease and risk factor surveillance in 2018 revealed that 22.3% of adults in China did not attain the minimum recommended level of physical activity outlined by the WHO[19]. Consequently, it is imperative to identify the factors contributing to the lack of physical exercise. Research has indicated that individuals' inability to engage in and maintain regular physical activity is partly attributable to a psychological inclination favoring immediate rewards over delayed, more substantial ones[20].

Delay discounting, a concept rooted in behavioral economics, quantifies an individual's preference between a smaller immediate reward and a larger delayed reward[21,22]. It characterizes the extent to which individuals assign value to the future, representing a process through which decision makers subjectively devalue future events[23]. Individuals with significantly delayed discounting tend to undervalue the future. For instance, individuals with high-delay discounting tend to opt for immediate rewards rather than waiting for larger, delayed rewards, even if the delayed option offers considerably greater benefits. Generally, as individuals await rewards over a longer duration, the perceived value of future rewards diminishes[24]. For instance, older individuals may opt to abstain from physical exercise because they perceive a life without exercise as uncomplicated, enjoyable, and comfortable, and they may not deem the future benefits of physical activity worthwhile. Delay discounting has been theorized to underlie several significant social behaviors, including addiction, obesity, and risky sexual behaviors[25]. As delay discounting increases, the value attributed to the future decreases. Delay aversion refers to aversion stemming from discounting the desired outcome, leading to the avoidance of delayed consequences due to negative emotional reactions[26]. The greater the degree of delay aversion, the more pronounced the aversion, resulting in heightened reluctance to opt for delayed outcomes[27]. Research has delved into delay discounting in various contexts, such as food consumption, exercise, smoking, and obesity[28], consistently demonstrating that increased delay discounting is associated with poorer health-related behaviors and outcomes. In studies concerning prediabetes, individuals exhibiting higher delay discounting tend to adopt unhealthy diets, engage in less physical activity, and demonstrate reduced drug adherence[29]. Previous research[27,30] on T2DM has also linked delay discounting with self-management behaviors, glycemic control, and physical activity, with higher levels of delay discounting being correlated with lower levels of physical activity[30].

The extant body of research on delay discounting and health behaviors has predominantly focused on developed countries, such as the United States[21,25,27,29]. Few studies have explored the relationship between delayed discounting and physical activity within the context of T2DM. Furthermore, the perspective of delay aversion has yet to be applied to the study of physical activity, and no investigations have been conducted on the correlation between delay discounting, delay aversion, and health-related aspects, such as physical activity, especially among Chinese adults diagnosed with T2DM. Therefore, examining the role of delay discounting and aversion in understanding the unwillingness of individuals with T2DM to engage in physical activity presents a novel approach. This study sheds light on physical activity among T2DM patients from a new perspective by exploring the association between delay discounting, delay aversion, and varying levels of physical activity intensity.

MATERIALS AND METHODS

Study population

This cross-sectional study recruited 400 patients aged 18 years and older who were all diagnosed with T2DM. This study was conducted at the People's Hospital of Linxia Hui Autonomous Prefecture in Gansu Province between February 2023 and June 2023. The eligibility criteria included individuals who were 18 years or older and had a clinical diagnosis of T2DM. The researchers apprised adults with T2DM of the study's significance and content, seeking willingness to participate. Those willing to participate were required to provide informed consent by signing the consent form.

Patients with T2DM who chose to participate were informed by the researchers regarding the approximate duration and necessary precautions for the in-person administration of the questionnaire, completion of the paper-based questionnaire, and performance of the HbA1c test for each participant. This study was approved by the Ethics Committee of the People's Hospital of Linxia Hui Autonomous Prefecture of Gansu Province (2022102101) and was conducted in accordance with ethical guidelines.

Measurement

Sample characteristics included age, sex, ethnicity, marital status, level of education, total annual household income, type of health insurance, and duration of diabetes. Age and diabetes duration were treated as continuous variables, with reported statistics including the mean, standard deviation, median, and interquartile range. Sex was categorized as either male or female, while ethnicity was categorized as Han, Hui, and other. Marital status was categorized as married or unmarried, and level of education was categorized as no formal schooling, primary school, junior high school, senior high

school/technical school, and college or higher education. The total annual household income levels were classified into three categories: RMB 0-34999, RMB 35000-74999, and RMB 75000 and above. The types of medical insurance were categorized as urban employee-based basic medical insurance (UEBMI), urban resident-based basic medical insurance (URBMI), and New Rural Cooperative Medical Insurance (NRCMI). The recruitment site was a general tertiary-care hospital.

In this study, the independent variables were delay discounting and delay aversion, both of which were assessed using the Quick Delay Questionnaire. This questionnaire comprises 10 self-reported items and serves to measure two distinct aspects of delay-related behaviors in adults: A 5-item measure of delay discounting and another 5-item measure of delay aversion. Scores were calculated independently for each subscale, with higher scores indicating a greater inclination toward discounting delays (*i.e.,* placing less emphasis on the future) and heightened aversion to delays (*i.e.,* experiencing negative emotions in response to delays)[31].

Outcome variables

The study outcomes primarily encompassed physical activity, which was assessed using the International Physical Activity Questionnaire-long form. This questionnaire assesses the nature of activities (*e.g.,* work, transportation, household gardening, leisure) and their respective intensities (walking, moderate intensity, and vigorous intensity). Participants were systematically queried about their physical activity experiences over the preceding 7 days, addressing work-related activities, transportation, household gardening, and leisure activities. Within each category, participants were prompted to provide details on the frequency (days per week) and daily cumulative duration (hours per day) of physical activity for the three distinct intensity levels. It is worth noting that any individual reporting a cumulative daily total of physical activity exceeding minutes (16 hours) was excluded from the analysis, and it is noteworthy that no participants in this study exceeded this threshold.

Metabolic equivalents (MET) were assigned specific values: 3.3 for walking, 3.0-6.0 for MET, and 8.0 for high-intensity physical activity (MET)[32]. The weekly level of physical activity for a particular intensity was calculated in Met-hours per week, accounting for the MET value corresponding to the intensity, weekly frequency (days per week), and daily duration (hours per day) of the activity. Specifically, the physical activity level for walking (Met-hours/week) was determined by aggregating walking activities at work, during transportation trips, and leisure. Moderate physical activity level (Met-hour/week) was calculated by combining moderate physical activity at work, cycling activity during transportation trips, household activity, and moderate-intensity leisure activity. Similarly, high-intensity physical activity level (Met-hour/week) was derived by summing high-intensity activities at work and during leisure activities.

Statistical analysis

A sample size of 400 was deemed sufficient to maintain 80% statistical power in the multivariate analysis. Specifically, this sample size provided ample statistical power to detect a minimum change of 10% in the R^2 value concerning the relationship between the primary independent variables (delay discounting and delay aversion) and physical activity while accounting for the contributions of covariates. This level of sensitivity aligns with Cohen's classification, which is capable of detecting small-to-medium effects ranging from 2% to 13% in R^2 values[33].

Sample characteristics, including counts, percentages, means, standard deviations, medians, and interquartile ranges were computed. The data were scrutinized to assess normality and independence, and chi-square tests were performed to ensure compliance with the assumptions underpinning the linear regression analysis. Interactions were initially explored for potential effects between delay discounting and ethnicity, as well as between delay aversion and ethnicity. However, these interactions did not yield statistically significant results. Consequently, the final multiple linear regression model was unstratified.

For statistical analysis, we used R version 4.3.1. Significance was assessed using a two-sided test at a significance level (α) of 0.05.

RESULTS

In our study population, the Cronbach's coefficients alpha for the delay discounting self-scale and delay aversion self-scale demonstrated good internal consistency, with values of 0.60 and 0.64, respectively.

Basic characteristics of the 400 participants

This study included 400 adults diagnosed with T2DM, with an average age of 57.5 years and median diabetes duration of 6.5 years. The participants' demographic breakdown revealed that 66.5% were male and 91.8% were married. In terms of ethnicity, 44.5% were identified as Han, 32.0% as Hui, 23.5% as other. The insurance coverage included 48.8% UEBMI, 19.0% URBMI, and 32.2% NRCMI. Educational level encompassed 22.8% with no formal schooling, 16.0% with primary school education, 18.0% with junior high school education, 18.7% with senior high school/technical school education, and 24.5% with a college degree or higher. Regarding total annual household income, 10.8% had an unknown income, 30.5% had incomes of 34900 yuan or below, 28.2% had incomes ranging from 35000 to 74900 yuan, and 30.5% had incomes of 75000 yuan and above (Table 1).

Table 1 Characteristics of the 400 participants

Sample characteristic	Mean (SD) or M (Q1, Q3) or *n* (%)
Age, yr	57.5 ± 10.9
Duration of diabetes, yr	6.5 (2.5, 12.0)
Sex	
Male	266 (66.5)
Female	134 (33.5)
Type of medical insurance	
UEBMI	195 (48.8)
URBMI	76 (19.0)
NRCMI	129 (32.2)
Ethnicity	
Han	178 (44.5)
Hui	128 (32.0)
Other	94 (23.5)
Marital status	
Married	367 (91.8)
Unmarried	33 (8.2)
Level of education	
No formal school	91 (22.8)
Primary school	64 (16.0)
Junior high school	72 (18.0)
Senior high school	75 (18.7)
College and above	98 (24.5)
Annual household income, yuan per year (1 yuan approximately equal to 0.155 USD)	
< 35000	122 (30.5)
35000-75000	113 (28.2)
> 75000	122 (30.5)
Unknown	43 (10.8)

UEBMI: Urban employee-based basic medical insurance; URBMI: Urban resident-based basic medical insurance; NRCMI: New rural cooperative medical insurance.

A multiple linear regression analysis of delay discounting with HbA1c and different intensity of physical activity (Met-hour/week)

In unadjusted linear regression models (linear regression results for unadjusted sample characteristics not shown in the table), delay discounting exhibited correlations with HbA1c (β = 0.016, 95%CI: -0.062 to -0.093). However, the association between walking activity level (β = 0.084, 95%CI: -1.172 to 1.341) and vigorous physical activity level (β = -0.062, 95%CI: -1.323 to 1.198) was not statistically significant. Notably, delayed discounting displayed a negative correlation with moderate physical activity levels (β = -2.428, 95%CI: -4.426 to -0.429), and this relationship was statistically significant. After adjusting for sample characteristics, the multiple linear regression models revealed associations between delay discounting and HbA1c (β = 0.024, 95%CI: -0.053 to 0.101). However, the relationship between walking activity level (β = 0.072, 95%CI: -1.192 to 1.336) and vigorous physical activity level (β = -0.065, 95%CI: -1.179 to 1.308) remained statistically non-significant. In contrast, the association between delayed discounting and moderate physical activity remained significant (β = -2.386, 95%CI: -4.370 to -0.401) (Table 2).

Table 2 The multiple linear regression analysis results regarding delay discounting, glycated hemoglobin, and different intensity levels of physical activity (Met-hour/week)

Sample characteristic	HbA1c β (95%CI)	Walking activity β (95%CI)	Moderate physical activity β (95%CI)	Vigorous physical activity β (95%CI)
Delay discounting	0.024 (-0.053, 0.101)	0.072 (-1.192, 1.336)	-2.386 (-4.370, -0.401)[a]	0.065 (-1.179, 1.308)
Age, yr	-0.024 (-0.047, -0.002)[a]	-0.086 (-0.452, 0.280)	-0.905 (-1.480, -0.330)[b]	-0.497 (-0.858, -0.137)[b]
Duration of diabetes, yr	-0.004 (-0.035, 0.027)	-0.038 (-0.550, 0.474)	0.489 (-0.315, 1.293)	-0.135 (-0.639, 0.368)
Sex				
Male	Reference	Reference	Reference	Reference
Female	-0.133 (-0.609, 0.343)	0.294 (-7.515, 8.104)	3.925 (-8.341, 16.190)	-6.832 (-14.518, 0.854)
Medical insurance type				
UEBMI	Reference	Reference	Reference	Reference
URBMI	0.269 (-0.474, 1.012)	-7.565 (-19.758, 4.627)	15.345 (-3.804, 34.494)	8.971 (-3.029, 20.971)
NRCMI	0.863 (0.140, 1.586)[a]	2.476 (-9.388, 14.340)	20.642 (2.009, 39.275)[a]	11.453 (-0.223, 23.130)
Ethnicity				
Han	Reference	Reference	Reference	Reference
Hui	-0.247 (-0.752, 0.259)	3.682 (-4.607, 11.972)	0.009 (-13.010, 13.028)	-0.231 (-8.389, 7.927)
Other	-0.452 (-1.012, 0.109)	-4.745 (-13.941, 4.451)	-1.869 (-16.311, 12.574)	7.126 (-1.924, 16.177)
Marital status				
Married	Reference	Reference	Reference	Reference
Unmarried	0.398 (-0.387, 1.182)	-4.171 (-17.038, 8.696)	15.021 (-5.188, 35.229)	-3.814 (-16.477, 8.850)
Level of education	-0.084 (-0.300, 0.133)	3.754 (0.209, 7.300)[a]	4.67 (-0.899, 10.239)	2.482 (-1.008, 5.971)
Annual household income, yuan per year				
< 35000	Reference	Reference	Reference	Reference
35000-75000	-0.26 (-0.837, 0.317)	-0.065 (-9.530, 9.400)	15.149 (0.283, 30.014)[a]	0.647 (-8.668, 9.962)
> 75000	-0.259 (-0.899, 0.380)	-0.908 (-11.394, 9.579)	-7.146 (-23.616, 9.324)	-6.052 (-16.373, 4.269)
Unknown	0.226 (-0.512, 0.965)	-2.252 (-14.364, 9.860)	0.591 (-18.432, 19.613)	-8.053 (-19.973, 3.867)
Constant	10.685 (8.288, 13.081)	34.193 (-5.120, 73.507)	84.431 (22.688, 146.175)	38.294 (-0.397, 76.985)

[a]$P < 0.05$.
[b]$P < 0.01$.
β-Standardized regression coefficient. UEBMI: Urban employee-based basic medical insurance; URBMI: Urban resident-based basic medical insurance; NRCMI: New rural cooperative medical insurance.

A multiple linear regression analysis of delay aversion with HbA1c, and different intensity of physical activity (Met-hour/week)

In the unadjusted linear regression models (linear regression results for unadjusted sample characteristics not shown in the table), delay aversion was associations with HbA1c (β = -0.008, 95%CI: -0.089 to 0.073), walking activity level (β = -0.749, 95%CI: -2.061 to 0.564), and vigorous physical activity level (β = -0.548, 95%CI: -1.866 to 0.770). However, the correlations were not statistically significant. Importantly, delay aversion displayed a negative correlation with moderate physical activity level (β = -3.781, 95%CI: -5.854 to -1.707), and this association was statistically significant. After adjusting for sample characteristics, the multiple linear regression model revealed associations between delay aversion and HbA1c (β = -0.002, 95%CI: -0.083 to 0.078), walking activity level (β = -0.724, 95%CI: -2.037 to 0.590), and vigorous physical activity level (β = -0.334, 95%CI: -1.629 to 0.960). Notably, these correlations were not statistically significant. Conversely, the association between delay aversion and moderate physical activity remained significant (β = -3.527, 95%CI: -5.578 to -1.476) (Table 3).

Table 3 The multiple linear regression analysis results regarding delay aversion, HbA1C, and different intensity levels of physical activity (Met-hour/week).

Sample characteristic	HbA1c β (95%CI)	Walking activity β (95%CI)	Moderate physical activity β (95%CI)	Vigorous physical activity β (95%CI)
Delay aversion	-0.002 (-0.083, 0.078)	-0.724 (-2.037, 0.590)	-3.527 (-5.578, -1.476)[b]	-0.334 (-1.629, 0.960)
Age, yr	-0.025 (-0.047, -0.003)[a]	-0.098 (-0.462, 0.266)	-0.885 (-1.454, -0.317)[b]	-0.504 (-0.863, -0.145)[b]
Duration of diabetes, yr	-0.003 (-0.035, 0.028)	-0.037 (-0.548, 0.473)	0.451 (-0.347, 1.248)	-0.135 (-0.638, 0.369)
Sex				
Male	Reference	Reference	Reference	Reference
Female	-0.122 (-0.598, 0.354)	0.582 (-7.213, 8.377)	4.213 (-7.958, 16.383)	-6.686 (-14.367, 0.994)
Medical insurance type				
UEBMI	Reference	Reference	Reference	Reference
URBMI	0.264 (-0.479, 1.008)	-7.588 (-19.760, 4.584)	15.757 (-3.247, 34.762)	8.954 (-3.039, 20.948)
NRCMI	0.849 (0.126, 1.573)[a]	2.121 (-9.720, 13.962)	20.374 (1.886, 38.861)[a]	11.273 (-0.395, 22.940)
Ethnicity				
Han	Reference	Reference	Reference	Reference
Hui	-0.259 (-0.763, 0.245)	3.528 (-4.728, 11.784)	0.622 (-12.268, 13.512)	-0.318 (-8.453, 7.817)
Other	-0.458 (-1.019, 0.103)	-4.872 (-14.050, 4.307)	-1.8 (-16.131, 12.531)	7.06 (-1.985, 16.104)
Marital status				
Married	Reference	Reference	Reference	Reference
Unmarried	0.389 (-0.396, 1.174)	-4.433 (-17.281, 8.416)	14.621 (-5.439, 34.682)	-3.944 (-16.605, 8.716)
Level of education	-0.088 (-0.305, 0.128)	3.639 (0.102, 7.176)[a]	4.625 (-0.898, 10.148)	2.422 (-1.063, 5.908)
Annual household income, yuan per year				
< 35000	Reference	Reference	Reference	Reference
35000-75000	-0.251 (-0.827, 0.326)	0.036 (-9.403, 9.475)	14.569 (-0.168, 29.306)	0.706 (-8.595, 10.006)
> 75000	-0.237 (-0.875, 0.402)	-0.291 (-10.751, 10.169)	-6.559 (-22.890, 9.772)	-5.74 (-16.046, 4.567)
Unknown	0.24 (-0.500, 0.979)	-1.77 (-13.875, 10.334)	1.584 (-17.315, 20.483)	-7.815 (-19.743, 4.112)
Constant	11.114 (8.726, 13.503)	46.666 (7.557, 85.776)	100.185 (39.122, 161.247)	44.574 (6.038, 83.110)

[a]$P < 0.05$.
[b]$P < 0.01$.
β-Standardized regression coefficient. UEBMI: Urban employee-based basic medical insurance; URBMI: Urban resident-based basic medical insurance; NRCMI: New rural cooperative medical insurance.

DISCUSSION

This study represents a pioneering investigation into the interplay between delay discounting and delay aversion, and their associations with varying levels of physical activity, particularly within the context of Chinese adults diagnosed with T2DM. Notably, this research contributes to the limited body of work exploring delay discounting in individuals with T2DM. Following adjustments for sample characteristics, multiple linear regression models revealed significant negative correlations between delay discounting and delay aversion with moderate physical activity levels. These findings suggest that individuals who place less emphasis on future rewards and are more inclined toward immediate gratification, as well as those who experience heightened negative emotions when rewards are delayed, tend to engage in less moderate physical activity.

In summary, this study provides novel insights into the relationship between delay discounting, delay aversion, and different levels of physical activity among Chinese adults with T2DM. Although no significant associations were found between delay discounting, delay aversion, and HbA1c in this study population, this represents the first endeavor to explore the interplay between delay discounting, delay aversion, different levels of HbA1c, and physical activity. These findings underscore the potential significance of delay discounting and delay aversion as influential factors affecting

participation in moderate physical activity within the T2DM population.

Furthermore, the results of this study underline the clinical relevance of the relationship between delay discounting, delay aversion, and moderate physical activity. Prior research has established that moderate to vigorous physical activity not only aids in glycemic control but also diminishes the risk of T2DM complications and overall mortality[7,13]. Therefore, understanding the roles of future value perception and delaying emotional responses is crucial when devising effective physical activity programs for individuals with T2DM.

This study implies that interventions aimed at promoting physical activity should consider individuals' delay discounting and delay aversion profiles and incorporate them into the design of interventions, including aspects such as goal setting, motivation strategies, and message framing. Identifying individuals with elevated levels of delay discounting, delay aversion, and tailoring interventions to address these factors is imperative for optimizing the effectiveness of physical activity interventions. Delay discounting and aversion have often been overlooked and underexplored in the development of intervention programs[30], but this study underscores their importance and suggests that they should be given due consideration in future physical activity plans, which could help sustain healthy behaviors within T2DM populations over time.

Investigating the relationships between delay discounting, delay aversion, and different levels of physical activity in the context of T2DM is a burgeoning area in diabetes health behavior research. Few studies or interventions have delved into this realm, with most examining cross-sectional associations between delay discounting, self-management behaviors, and HbA1c[34,35]. Notably, a cross-sectional study in the United States linked delay discounting and aversion with self-management behaviors and quality of life among adults with T2DM[27]. Understanding the roles of delay discounting and delay aversion opens new perspectives for institutions and governments in shaping policies regarding health behaviors of individuals with diabetes.

Furthermore, it is worth highlighting that both delay discounting and delay aversion are modifiable factors[27]. Although relatively few studies have explored them as adjustable targets for improving health behaviors, developing interventions to address these factors may bolster participation in physical activity. One approach with a proven track record for reducing delay discounting is episodic future thinking[36,37], which involves vividly envisioning positive future events. Epstein *et al*[38] employed episodic future thinking in clustering interventions pertinent to prediabetes and subsequently analyzed alterations in delay discounting, HbA1c, and levels of physical activity. Stein *et al*[39] documented a significant reduction in delay discounting among adults with a heightened risk of T2DM through episodic future thinking. This method will be utilized in future studies to diminish delay in discounting and enhance engagement in physical activities.

Despite its collection of primary data from a substantial cohort of adults diagnosed with T2DM, this study exhibits specific limitations. Notably, it lacks a chronological sequence, precluding the establishment of any causal relationships, thereby characterizing the study as cross-sectional. Additionally, it should be noted that the recruitment of individuals with T2DM was confined to a tertiary care hospital situated in an ethnically diverse region of northwest China. Consequently, the applicability of these findings may be restricted. Furthermore, the quantification of physical activity relied on self-reporting rather than a direct measurement methodology, potentially introducing recall bias into the study.

In conclusion, the influence of delay discounting and aversion on physical activity in the context of T2DM has substantial implications for both research and policy. Given the limited existing evidence, further research is warranted to comprehensively investigate the roles of delay discounting and aversion in relation to different levels of physical activity and diabetes outcomes.

CONCLUSION

This study uncovered a correlation between elevated delay discounting and increased delay aversion with reduced levels of moderate physical activity in a cohort of adults diagnosed with T2DM. Findings suggest the potential involvement of delay discounting and delay aversion in the context of moderate physical activity. Moreover, delay discounting and delay aversion may affect the participation of moderate physical activity. Therefore, this study recommends that health administration and governments consider delay discounting and delay aversion when formulating behavioral intervention strategies and treatment guidelines involving physical activity for patients with T2DM, possibly increasing participation in physical activity. So as to prevent and reduce the complications of diabetes and severity of various chronic non-communicable diseases, thereby improving the quality of life. Future investigations should aim to provide a markedly comprehensive understanding of the intricate interplay among delay discounting, delay aversion, physical activity, and diabetes-related outcomes. Moreover, there is a need to develop targeted interventions designed to address delay discounting and aversion. Such interventions could be instrumental in fostering participation in and sustaining moderate physical activity among individuals with T2DM, thereby contributing to improved diabetes outcomes and overall health.

ARTICLE HIGHLIGHTS

Research background

Physical inactivity is the fourth leading cause of mortality worldwide and is a modifiable risk factor. Physical activity not only proves effective in managing blood glucose levels and reducing risk factors for cardiovascular disease in individuals

with type 2 diabetes mellitus (T2DM) but also serves as a preventive measure against or delay in the onset of diabetes-related complications. In China, 22.3% of adults do not meet the minimum level of physical activity recommended by the World Health Organization.

Research motivation

Research has indicated that individuals' inability to engage in and maintain regular physical activity is partly attributable to a psychological inclination favoring immediate rewards over delayed, more substantial ones.

Research objectives

To investigate the relationship between delay discounting, delay aversion, glycated hemoglobin (HbA1c), and various levels of physical activity in Chinese adults diagnosed with T2DM.

Research methods

In 2023, 400 adults with T2DM were recruited from the People's Hospital of Linxia Hui Autonomous Prefecture of Gansu Province. A face-to-face questionnaire was used to gather demographic data and details on physical activity, delay discounting, and delay aversion. In addition, HbA1c levels were measured in all 400 participants. Multiple linear regression models were utilized to assess the relationship between delay discounting, delay aversion, and HbA1c levels, along with the intensity of different physical activities measured in met-hours per week.

Research results

After controlling for sample characteristics, delay discounting was negatively associated with moderate physical activity (β = -2.386, 95%CI: -4.370 to -0.401). Similarly, delay aversion was negatively associated with the level of moderate physical activity (β = -3.527, 95%CI:-5.578 to -1.476) in the multiple linear regression model, with statistically significant differences.

Research conclusions

Elevated delay discounting and increased delay aversion correlated with reduced levels of moderate physical activity. Result suggests that delay discounting and aversion may influence engagement in moderate physical activity. This study recommends that health administration and government consider delay discounting and delay aversion when formulating behavioral intervention strategies and treatment guidelines involving physical activity for patients with T2DM, which may increase participation in physical activity.

Research perspectives

It is worth highlighting that both delay discounting and delay aversion are modifiable factors, developing interventions to address these factors may bolster participation in physical activity. One approach with a proven track record for reducing delay discounting is episodic future thinking, which involves vividly envisioning positive future events. This method will be utilized in future studies to diminish delay in discounting and enhance engagement in physical activities.

FOOTNOTES

Author contributions: An YD and Zhang ZL designed the study and wrote the first version of the manuscript, and performed the statistical analyses; Ma GX, Cai XK, Yang Y, and Wang F were participated in recruitment and examination of the subjects and/or collection of data; all authors have approved the manuscript.

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ORIGINAL ARTICLE

Observational Study

Correlation of periodontal inflamed surface area with glycated hemoglobin, interleukin-6 and lipoprotein(a) in type 2 diabetes with retinopathy

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Abstract

BACKGROUND

The two-way relationship between periodontitis and type 2 diabetes mellitus (T2DM) is well established. Prolonged hyperglycemia contributes to increased periodontal destruction and severe periodontitis, accentuating diabetic complications. An inflammatory link exists between diabetic retinopathy (DR) and periodontitis, but the studies regarding this association and the role of lipoprotein(a) [Lp(a)] and interleukin-6 (IL-6) in these conditions are scarce in the literature.

AIM

To determine the correlation of periodontal inflamed surface area (PISA) with glycated Hb (HbA1c), serum IL-6 and Lp(a) in T2DM subjects with retinopathy.

METHODS

This cross-sectional study comprised 40 T2DM subjects with DR and 40 T2DM subjects without DR. All subjects were assessed for periodontal parameters [bleeding on probing (BOP), probing pocket depth, clinical attachment loss (CAL), oral hygiene index-simplified, plaque index (PI) and PISA], and systemic parameters [HbA1c, fasting plasma glucose and postprandial plasma glucose, fasting lipid profile, serum IL-6 and serum Lp(a)].

RESULTS

The proportion of periodontitis in T2DM with and without DR was 47.5% and 27.5% respectively. Severity of periodontitis, CAL, PISA, IL-6 and Lp(a) were higher in T2DM with DR group compared to T2DM without DR group. Sig-

nificant difference was observed in the mean percentage of sites with BOP between T2DM with DR (69%) and T2DM without DR (41%), but there was no significant difference in PI ($P > 0.05$). HbA1c was positively correlated with CAL ($r = 0.351$, $P = 0.001$), and PISA ($r = 0.393$, $P \leq 0.001$) in study subjects. A positive correlation was found between PISA and IL-6 ($r = 0.651$, $P < 0.0001$); PISA and Lp(a) ($r = 0.59$, $P < 0.001$); CAL and IL-6 ($r = 0.527$, $P < 0.0001$) and CAL and Lp(a) ($r = 0.631$, $P < 0.001$) among study subjects.

CONCLUSION

Despite both groups having poor glycemic control and comparable plaque scores, the periodontal parameters were higher in DR as compared to T2DM without DR. Since a bidirectional link exists between periodontitis and DM, the presence of DR may have contributed to the severity of periodontal destruction and periodontitis may have influenced the progression of DR.

Key Words: Type 2 diabetes mellitus; Periodontitis; Periodontal inflamed surface Area; Glycated Hb; Diabetic retinopathy

Core Tip: Periodontal inflamed surface area (PISA) estimates the periodontal inflammatory burden. Prolonged hyperglycemia contributes to increased periodontal destruction and accentuates diabetic complications. An inflammatory link may exist between diabetic retinopathy (DR) and periodontitis. This study assessed correlation between PISA with glycated Hb (HbA1c), interleukin-6 (IL-6) and lipoprotein(a) [Lp(a)] in type 2 diabetes mellitus (T2DM) subjects with and without DR. Significant positive correlation between PISA with HbA1C, IL-6 and Lp(a) were observed. Proportion and severity of periodontitis, PISA, IL-6 and Lp(a) were higher in DR compared to T2DM without DR. Presence of DR may have contributed to the severity of periodontitis and periodontitis may have influenced the progression of DR.

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INTRODUCTION

Diabetes mellitus (DM) is a multifaceted metabolic disorder characterized by impaired glucose tolerance and hyperglycemia. Currently, 537 million people are living with diabetes, which is predicted to rise to 643 million by 2030 and 784 million by 2045[1]. Type 2 DM (T2DM) occurs when there is a progressive loss of insulin secretion on the background of insulin resistance. Secondary pathophysiologic changes in diabetes lead to the development of microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular (ischemic heart disease, peripheral vascular disease, and cerebrovascular disease) complications. Periodontitis has been recognized as the "sixth complication" of diabetes[2].

Periodontitis is a microbially-associated, host-mediated inflammation of the supporting tissues of teeth characterized by the presence of periodontal pockets, clinical attachment loss (CAL), gingival recession, and progressive destruction of periodontal structures[3]. The periodontal pocket acts as a portal for the entry of microorganisms into the systemic circulation, leading to subclinical systemic inflammation. Periodontitis induced bacteremia and inflammatory response depends on the area of inflamed periodontal tissue. Periodontal inflamed surface area (PISA) counts the surface area of bleeding periodontal pocket epithelium and assesses the inflammatory burden[4]. A two-way relationship exists between periodontitis and diabetes. Poor glycemic control, longer duration and complications of DM lead to periodontal disease (PD) severity[5]. Systemic inflammation reduces insulin sensitivity, increases insulin resistance, and thus adversely affects the glycemic status, which in turn increases the risk of complications of DM[6].

Diabetic retinopathy (DR) is one of the most common microvascular complications of DM, and it is the progressive dysfunction of the retinal blood vessels caused by chronic hyperglycemia[7]. It is one of the leading causes of blindness. Approximately one in three people with diabetes have DR and one in ten will develop a vision – threatening form of the disease[8]. DR is perceived as a vascular and neurodegenerative disease. Inflammation plays a crucial role in the development of the early and late stages of DR[9].

Lipoprotein(a) [Lp(a)] is involved in the development of atherothrombosis and the activation of acute inflammation, exerting a proatherogenic and hypofibrinolytic effect[10]. Since capillary occlusion is a frequent finding in DR, the factor Lp(a) has an important role in the development and progression of DR. Lp(a) is susceptible to oxidative modifications, leading to the formation of pro-inflammatory and pro-atherogenic oxidized phospholipids. It has been reported that PD leads to elevated levels of lipoproteins and inflammatory mediators in the serum and gingival crevicular fluid[11]. Elevated inflammatory mediators and lipoproteins in diabetic patients with PD may contribute to retinal blood vessel damage, leading to DR.

Studies regarding the relationship between DR and PD and the role of Lp(a) and interleukin-6 (IL-6) in these conditions are scarce in the literature. Therefore, the objectives of the present study were: (1) To compare the proportion and severity of periodontitis; and (2) To correlate CAL and PISA with glycemic status, serum IL-6, and Lp(a) in T2DM subjects with and without DR.

MATERIALS AND METHODS

This cross-sectional study was carried out by the Department of Periodontics, Government Dental College, Calicut in association with the diabetic clinic and Dept of Ophthalmology, Government Medical College, Calicut. The participants were T2DM patients attending the diabetic clinic of Government Medical College, Calicut. T2DM subjects with and without DR in the age group between 30-75 years were included in the study. Exclusion criteria were: History of intraocular surgery or previous laser photocoagulation, patients who were already on lipid-lowering drugs, known systemic diseases and conditions, pregnant and lactating mothers, psychiatric illness, systemic antibiotics within six months, periodontal therapy (scaling and root planing or surgery) within the past one year.

A total of 80 T2DM subjects (40 with DR and 40 without DR) were randomly selected. This study was approved by the Institutional Ethics Committee, Government Dental College Calicut (IEC No: 149/2019/DCC dated 14-11-2019). Informed consent was obtained from all subjects and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. The duration of the study was 18 months (from 30/05/2020 to 30/11/2021).

Clinical examination and assessment of DR

All subjects were evaluated using a detailed questionnaire including personal information, socio-demographic characteristics, medical history, oral hygiene practices, history of diabetes, duration of diabetes and diabetic complications, and drug allergy. Diagnosis of DR was done by dilated fundoscopy, performed by an ophthalmologist and its severity (mild, moderate, and severe) was assessed based on the International Clinical Diabetic Retinopathy Disease Severity Scale[12].

Biochemical variables

Biochemical variables were assessed using peripheral blood samples collected by venipuncture from the ante cubital fossa in the same period of clinical examination. The parameters included glycated Hb (HbA1c), fasting plasma glucose, postprandial plasma glucose, fasting lipid profile (FLP), IL-6, and Lp(a).

Oral and periodontal examination

Plaque index (PI), oral hygiene index-simplified (OHI-S), percentage of sites with bleeding on probing (BOP), gingival recession (GR), probing pocket depth (PPD), CAL, and PISA were recorded. All periodontal assessments were carried out by a qualified examiner (Nusreen Jamal Thazhe Poyil).

William's periodontal probe was used to assess PPD, GR, and CAL at six sites per tooth. The periodontal status was recorded as no/mild, moderate, and severe periodontitis, based on the CDC criteria (CDC 2012 update)[13].

PISA was calculated using a Microsoft Excel spreadsheet available from the website: www.parsprototo.info. CAL, GR, and BOP on six sites for each tooth were entered in this spreadsheet. Mean CAL and GR for each tooth were computed and converted into periodontal epithelial surface area (PESA). PISA for a particular tooth was measured by multiplying PESA for that tooth with the percentage of sites with BOP. PISA (mm^2) per subject was estimated by adding PISA around each tooth.

Statistical analysis

mean ± SD and frequency were computed for quantitative and qualitative data respectively. Unpaired *t*-test was done to evaluate the quantitative variables [age, duration of diabetes, BOP, OHI-S, PI, PPD, CAL, HbA1c, fasting blood glucose, postprandial blood glucose, FLP, IL-6, Lp(a) and PISA] between T2DM patients with and without DR. The χ^2 test analyzed qualitative data such as oral hygiene practices, past smoking status, severity of DR, proportion, and severity of periodontitis. Mean CAL and PISA were analyzed between nonproliferative, proliferative type 2 DR and T2DM without DR by one-way ANOVA test with *post-hoc* adjustment (Bonferroni test). Correlation between PISA and HbA1c, PISA and IL-6, PISA and Lp(a), CAL and HbA1c, CAL and IL-6, and CAL and Lp(a) were done by Pearson correlation test.

RESULTS

Socio-demographic, behavioral, and clinical characteristics of the study subjects are given in Table 1. No significant difference was observed regarding the age, educational level and smoking status between T2DM subjects with and without DR. A significant difference in the percentage distribution of gender between the two groups with a male predominance in DR group ($P = 0.001$) was observed. The mean duration of T2DM ($P = 0.005$), mean debris score ($P < 0.001$), OHI-S score ($P = 0.02$) and Decay, missing and filled Teeth score ($P = 0.04$) were significantly higher in DR group, but there was no difference in PI ($P = 0.19$) and calculus index ($P = 0.36$).

The distribution of biochemical and periodontal variables between T2DM with and without DR group are displayed in Table 2. A significant difference was observed in the HbA1c ($P < 0.005$), serum IL-6 ($P < 0.001$), Lp(a) ($P < 0.001$), HDL ($P < 0.001$), low-density lipoprotein (LDL; $P = 0.025$), very LDL ($P = 0.005$) and triglyceride ($P = 0.004$) levels between the

Table 1 Socio-demographic, behavioral, and clinical characteristics of study subjects

Variables		T2DM with DR (*n* = 40)	T2DM without DR (*n* = 40)	*P* value
Age, yr (mean ± SD)		53.85 ± 6.86	54.03 ± 10.75	0.093
Gender frequency, *n* (%)	Male	31 (77.5)	17 (42.5)	0.001
	Female	9 (22.5)	23 (57.5)	
Education frequency, *n* (%)	Illiterate	0	1 (2.5)	0.172
	Primary school	5 (12.50)	4 (10)	
	Middle school	21 (52.50)	12 (30.0)	
	High school	11 (27.50)	21 (52.5)	
	Diploma and above	3 (7.5)	2 (5.0)	
Socioeconomic status frequency, *n* (%)	APL	25 (62.5)	19 (47.5)	0.178
	BPL	15 (37.5)	21 (52.5)	
Behavioural				
Smoking status, *n* (%)	Current smoker	1 (2.5)	1 (2.5)	0.49
	Ex-smoker	5 (12.5)	2 (5.0)	
	Non-smoker	34 (85.0)	37 (92.5)	
Frequency of teeth cleaning (daily), *n* (%)	Once	23 (57.5)	19 (47.5)	0.44
	Twice	17 (42.5)	20 (50.0)	
	After every meal	0 (0)	1 (2.5)	
Clinical (mean ± SD)				
Duration of DM (yr)		11.94 ± 6.83	8.07 ± 4.87	0.005[a]
DI-S		1.78 ± 0.69	1.22 ± 0.54	< 0.001[a]
CI-S		1.15 ± 0.79	1.00 ± 0.67	0.36
OHI-S		2.90 ± 1.38	2.22 ± 1.12	0.02[a]
PI		1.72 ± 0.57	1.54 ± 0.62	0.19
DMFT		7.48 ± 4.08	5.53 ± 4.45	0.04[a]

[a]*P* <0.05.

T2DM: Type 2 diabetes mellitus; DR: Diabetic retinopathy; APL: Above the poverty line; BPL: Below the poverty line; DM: Diabetes mellitus; CI-S: Calculus index-simplified; DI-S: Debris index-simplified; DMFT: Decayed, missing, filled teeth; OHI-S: Oral hygiene index-simplified; PI: Plaque index.

groups, but no significant difference was seen in the total cholesterol levels ($P = 0.254$).

The mean difference in PPD ($P < 0.001$), CAL ($P < 0.001$), PISA ($P < 0.001$) and percentage of sites with BOP ($P < 0.001$) between T2DM with DR group and T2DM without DR group was significant.

The proportion of periodontitis in T2DM with DR and in T2DM without DR was 47.5% and 27.5% respectively. Significant difference was observed in the severity of PD among the groups ($P = 0.05$; Figure 1). The proportion of mild periodontitis was higher in T2DM without DR (75%) as compared to T2DM with DR (52.5%) whereas moderate periodontitis was significantly higher among DR group (40%) than T2DM without DR (25%). No subjects had severe periodontitis in T2DM without retinopathy.

The mean difference in CAL and PISA between T2DM with nonproliferative, proliferative retinopathy group and T2DM without retinopathy groups was significant ($P < 0.001$). Bonferroni *post-hoc* adjustment revealed a significant difference between T2DM without retinopathy and nonproliferative retinopathy, and proliferative retinopathy groups ($P < 0.001$; Tables 3 and 4).

A statistically significant positive correlation was observed between PISA and HbA1c ($r = 0.393$, $P < 0.001$; Figure 2A), PISA and IL-6 ($r = 0.651$, $P < 0.001$; Figure 2B), PISA and Lp(a) ($r = 0.59$, $P < 0.001$; Figure 2C) among all study subjects. A statistically significant positive correlation was seen between CAL and HbA1c ($r = 0.351$, $P = 0.001$; Figure 2D), CAL and IL-6 ($r = 0.527$, $P < 0.001$; Figure 2E), CAL and Lp(a) ($r = 0.631$, $P < 0.001$; Figure 2F) in all study subjects.

Table 2 Comparison of biochemical and periodontal variables between groups

Variables	T2DM with DR (*n* = 40)	T2DM without DR (*n* = 40)	*P* value
HbA1c (%)	8.67 ± 1.81	7.64 ± 1.28	0.004[a]
FPG (mmol/L)	8.66 ± 2.67	8.62 ± 3.58	0.95
PPG (mmol/L)	12.22 ± 4.21	11.95 ± 4.05	0.77
IL-6 (pg/mL)	30.52 ± 16.57	13.47 ± 4.15	< 0.001[a]
Lp(a) (mg/dL)	27.76 ± 18.82	11.45 ± 9.08	< 0.001[a]
Total cholesterol (mmol/L)	4.93 ± 0.90	4.70 ± 0.89	0.254
HDL (mmol/L)	1.41 ± 0.34	1.78 ± 0.36	< 0.001[a]
LDL (mmol/L)	2.80 ± 0.93	2.31 ± 0.97	0.025 [a]
VLDL (mmol/L)	0.76 ± 0.28	0.61 ± 0.19	0.005 [a]
TG (mmol/L)	1.67 ± 0.61	1.32 ± 0.41	0.004 [a]
Periodontal variables			
PPD (mm)	3.87 ± 0.93	3.08 ± 0.66	< 0.001[a]
CAL (mm)	4.50 ± 1.49	3.43 ± 0.85	< 0.001[a]
CAL, ≤ 3 mm	36.14 ± 22.81	63.88 ± 26.52	< 0.001[a]
CAL, 4 to 5 mm	40.62 ± 15.56	28.91 ± 18.55	0.003[a]
CAL, ≥ 6 mm	23.42 ± 24.29	8.44 ± 11.99	0.001[a]
BOP (% of sites)	69.33 ± 23.36	40.95 ± 25.47	< 0.001[a]
PISA (mm^2)	1570.559 ± 759.89	789.79 ± 589.34	< 0.001[a]
Periodontitis, *n* (%)	19 (47.5)	11 (27.5)	0.065

[a]*P* < 0.05.

A significant difference among subjects with type 2 diabetes mellitus in glycated hemoglobin, interleukin-6, lipoprotein(a), high density lipoprotein, low density lipoprotein, very low-density lipoprotein, triglyceride, periodontal disease, clinical attachment loss (CAL), CAL ≤ 3 mm, CAL ≥ 6 mm, bleeding on probing, periodontal inflamed surface area, and severity of periodontitis. T2DM: Type 2 diabetes mellitus; DR: Diabetic retinopathy; HbA1c: Glycated hemoglobin; FPG: Fasting plasma glucose; PPG: Postprandial plasma glucose; Il-6: Interleukin-6; Lp(a): Lipoprotein(a); HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low-density lipoprotein; TG: Triglyceride; PPD: Probing pocket Depth; CAL: Clinical attachment loss; BOP: Bleeding on probing; PISA: Periodontal inflamed surface area.

Table 3 Comparison of clinical attachment loss and periodontal inflamed surface area among nonproliferative, proliferative and no retinopathy in type 2 diabetes mellitus patients

Variable	Non-proliferative diabetic retinopathy	Proliferative diabetic retinopathy	Diabetic without retinopathy	*P* value
Mean CAL (mm, 95%CI)	4.1 (3.66-4.72)	4.87 (4.00-5.75)	3.43 (3.15-3.70)	< 0.001[a]
Mean PISA (mm^2, 95%CI)	1380.87 (1087.12-1674.63)	1802.39 (1392.62-2212.17)	789.79 (601.31-978.27)	< 0.001[a]

[a]*P* < 0.05.

A significant difference in clinical attachment loss and periodontal inflamed surface area among non-proliferative, proliferative and no diabetic retinopathy. CAL: Clinical attachment loss; PISA: Periodontal inflamed surface area.

DISCUSSION

In the present study, the percentage of males was higher in DR group as compared to T2DM without DR and it is in accordance with the findings of Deshpande *et al*[14]. DR is often preceded by neurodegenerative changes and females may have some protection from, or resistance to, these changes relative to males[15]. This may be attributed to the low frequency of females in the current study.

The duration of diabetes was significantly longer in DR group (11.94 ± 6.83 years) in contrast to T2DM without DR (8.07 ± 4.87 years). Even though both the study groups had poor glycemic control, the HbA1c values were significantly higher in the DR group (8.67 ± 1.8) as compared to T2DM without DR (7.64 ± 1.2). Longer duration of diabetes and

Table 4 Bonferroni *post-hoc* adjustment

Dependent variable	Group	Group	Mean difference (95%CI)	*P* value	*F* statistic
Mean CAL	1	2	-0.68 (-1.61, 0.25)	0.23	9.58
		3	0.76 (-0.01, 1.54)	0.05[a]	
	2	3	1.44 (0.61, 2.28)	< 0.001[a]	
Mean PISA	1	2	-421.52 (-940.717, 97.681)	0.15	15.65
		3	591.08 (157.464, 1024.697)	0.004[a]	
	2	3	1012.59 (548.94, 1476.258)	< 0.001[a]	

[a]$P < 0.05$.

A significant difference in clinical attachment loss and periodontal inflamed surface area among non-proliferative, proliferative and no diabetic retinopathy. Group 1: Nonproliferative diabetic retinopathy; Group 2: Proliferative diabetic retinopathy; Group 3: Type 2 diabetes without retinopathy. CAL: Clinical attachment loss; PISA: Periodontal inflamed surface area.

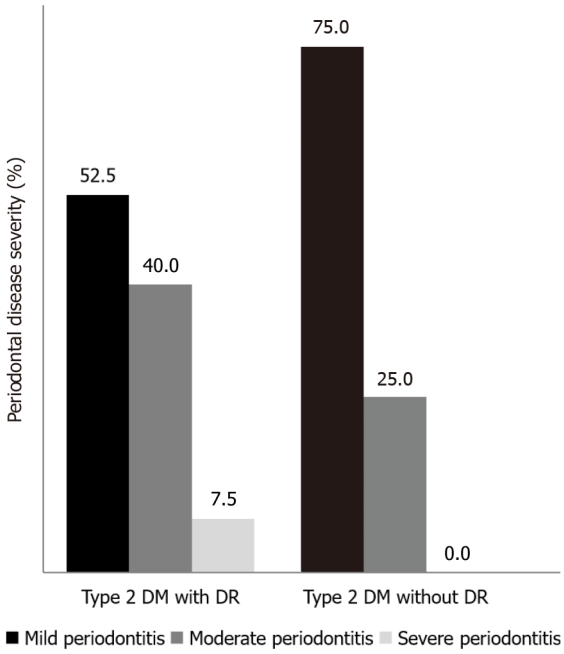

Figure 1 Proportion of periodontal disease severity among subjects in type 2 diabetes mellitus with and without diabetic retinopathy. DM: Diabetes mellitus; DR: Diabetic retinopathy.

increased glycemic burden are positively associated with the incidence of DR[16].

It is interesting to note that plaque score was similar in both groups, but the mean DI-S score, OHI-S score and DMFT scores were significantly higher in DR group as compared to T2DM group without DR. This may be due to the poor glycemic control and associated periodontal inflammation in DR which in turn could have adversely affected the ability to maintain good oral hygiene. Sadzeviciene *et al*[17] reported that a correlation exists between complications inherent to DM, such as DR or nephropathy, and an increased degree of periodontal inflammation.

T2DM with DR showed a higher percentage of periodontitis (47.5%) as compared to T2DM without DR (27.5%) in the present study. Although Amiri *et al*[18] in 2014 indicated a probable relationship between retinal microvascular complications in diabetes and PD, to the best of our knowledge, no studies have been conducted to compare the percentage of periodontitis between T2DM with and without DR. In this study, T2DM with DR group had a higher mean value of PPD and CAL. This observation was in accordance with the findings of Sadzeviciene *et al*[17] in 2005 who reported increased periodontal breakdown in the presence of microvascular complications of T2DM. Similar to this, Adhenkavil Radhakrishnan *et al*[19] in 2022 reported a higher frequency of periodontitis in diabetic foot patients. In contrast, Bridges *et al*[20] in 1996 opined that there was no association between glycemic control and periodontal variables.

DR group showed a higher percentage of sites with CAL ≥ 6 mm and a higher percentage of sites with moderate/severe periodontitis as compared to T2DM without DR. This showed that the severity of periodontitis was more in T2DM with DR. Anil *et al*[21] reported that the uncontrolled T2DM group with microvascular complications had the highest percentage of sites with CAL ≥ 6 mm than the uncontrolled T2DM group without microvascular complications and controlled T2DM group. Inflammatory cytokines and Advanced Glycation End products in hyperglycemic state induce

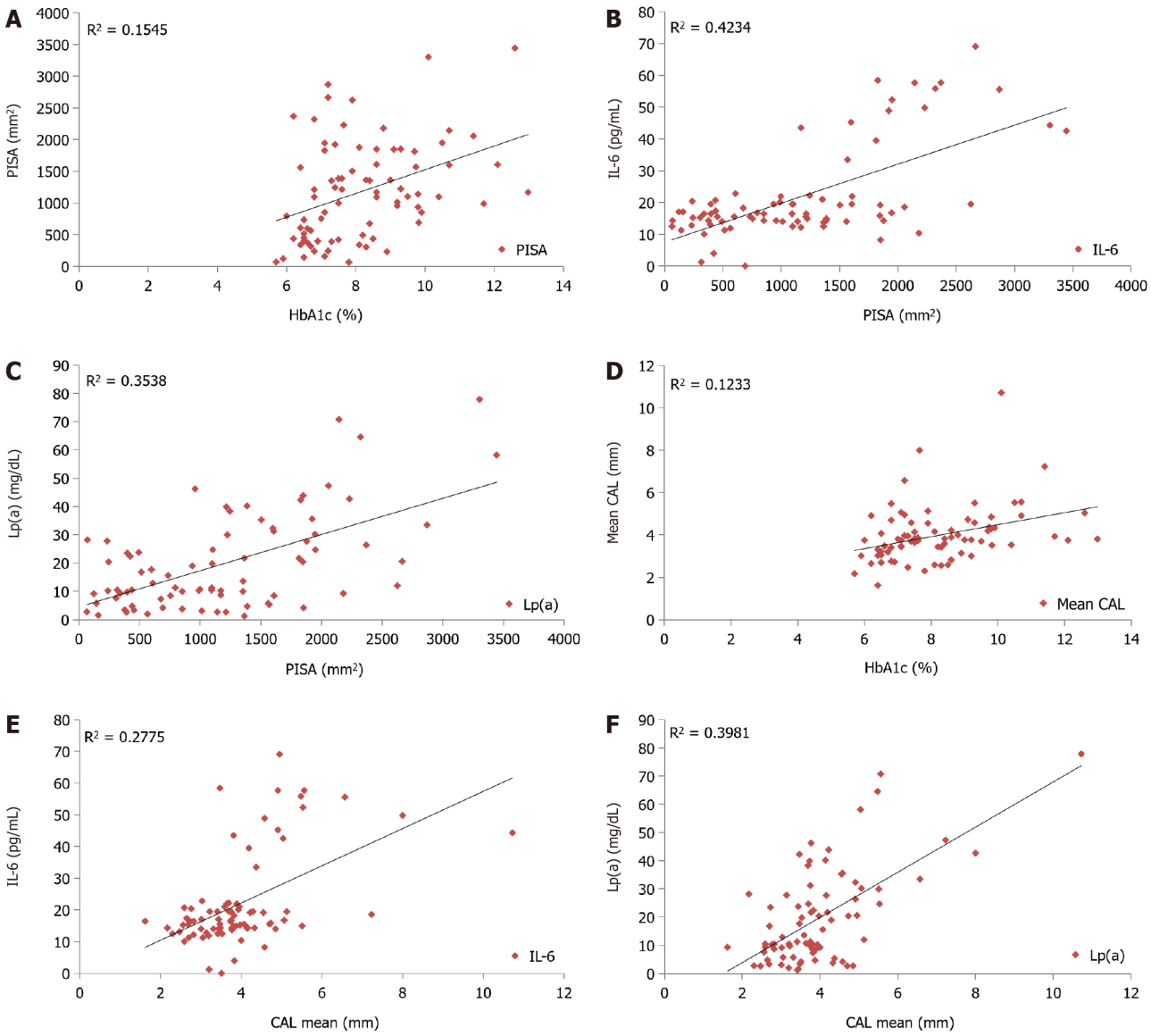

Figure 2 Correlation of periodontal inflamed surface area, and clinical attachment loss between interleukin-6, lipoprotein(a) and glycated hemoglobin. A: A statistically significant positive correlation had been found between the periodontal inflamed surface area and glycated hemoglobin (HbA1c) among study subjects (Pearson correlation coefficient 0.393, *P* value < 0.001); B: A positive correlation had been found between serum interleukin-6 (IL-6) and mean periodontal inflamed surface area among study subjects (Pearson correlation coefficient 0.651, *P* < 0.001); C: Positive correlation had been found between serum lipoprotein(a) [Lp(a)] and mean periodontal inflamed surface area among study subjects (Pearson correlation coefficient 0.59, *P* < 0.001); D: A statistically significant positive relation had been found between the mean clinical attachment loss (CAL) and HbA1c in type 2 diabetic mellitus subjects (Pearson correlation coefficient 0.351, *P* = 0.001); E: A positive correlation had been found between serum IL-6 and mean CAL among study subjects (Pearson correlation coefficient 0.527, *P* < 0.0001); F: Positive correlation had been found between serum Lp(a) and mean CAL among study subjects (Pearson correlation coefficient 0.631, *P* < 0.001). HbA1c: Glycated hemoglobin; PISA: Periodontal inflamed surface area; IL-6: Interleukin-6; Lp(a): Lipoprotein(a); CAL: Clinical attachment loss.

neutrophils to create oxidative stress that accentuates alveolar bone loss by acting on osteoclasts, resulting in increased attachment loss and severity of periodontitis in diabetic complications[22].

The link between DR and periodontitis may be possibly related to the presence of inflammation. NLRP3 (nucleotide-binding domain and leucine-rich repeat receptor containing a pyrin domain 3), is a protein responsible for several intracellular signalling events in the inflammatory mechanism. NLRP3 is not only involved in the pathogenesis of periodontitis but is also present in the retina of patients with progressive DR[23,24].

It is evident from this study that as the severity of DR increases the severity of periodontal breakdown increases. Proliferative DR had higher CAL compared to non-proliferative DR. A positive correlation was observed between CAL and HbA1c in T2DM with and without DR and these observations are comparable to the report of H R *et al*[25] in 2018. They reported that the severity of PD strongly correlated with HbA1c in T2DM with DR patients.

BOP is the earliest indicator of periodontal inflammation. The percentage of sites with BOP was 69% and 40.95% respectively in T2DM with DR and T2DM without DR. Severe periodontal inflammation in T2DM with DR may be accounted for increased bleeding sites. Consistent with this, Anil *et al*[21] reported 79%of sites with BOP in uncontrolled T2DM group with microvascular complications. Zoellner *et al*[26] reported that histologically, the microvascular pathological conditions of gingivitis and retinopathies are similar; both are described as microvascular angiopathies with

oedema, vascular proliferation and tortuosity, haemorrhaging, and membrane thickening. Lp(a) exerts antifibrinolytic and prothrombotic effects, which may contribute to the increased BOP in DR patients. Microvascular pathological conditions of retinopathy might have influenced the increased BOP and further studies are needed to confirm this.

Periodontitis induces systemic inflammatory burden by the ingress of inflammatory mediators. Tools such as CAL and PPD for grading periodontitis are linear measurements that do not adequately estimate the inflammatory load induced by periodontitis. So, in this study inflammatory burden of periodontitis was assessed by PISA. PISA shows the surface area of bleeding pocket epithelium[4], used to assess the periodontal inflammation. In this study T2DM with DR group had a higher mean PISA (1570 mm^2) than T2DM without DR (789 mm^2). This is in accordance with studies by Anil *et al* [21] and Lindner *et al*[27]. Nesse *et al*[4] in 2008 observed a higher PISA score with upper and lower limits of 0 and 1087 mm^2 respectively, in T2DM with periodontitis. Estimated PISA values in this study corroborate with the inflammatory link between periodontitis and DR. A positive correlation had been obtained between PISA and HbA1c in T2DM with DR and without DR which is similar to the report of Adhenkavil Radhakrishnan *et al*[19] in 2022. They reported a dose-response relationship exists between PISA and HbA1c and showed that an increase in PISA of 50.77 mm^2 was associated with a 1% increase in HbA1c. Nesse *et al*[4] in 2008 opined that a change in PISA of 333 mm^2 was associated with a 1% increase in HbA1c independent of other factors. From this, it is evident that periodontal inflammation could have influenced the HbA1c level in T2DM with and without DR.

The inflammatory response in periodontitis is characterized by dysregulated secretion of host-derived inflammatory mediators which may provoke systemic inflammation, enhance hyperglycemia, and insulin resistance and exacerbate complications in T2DM. In this study, serum levels were significantly high in retinopathy (30.52 pg/mL) when compared to T2DM without DR (13.47 pg/mL). Similar findings are reported by Quevedo-Martínez *et al*[28]. In this study, the presence of a higher percentage of periodontitis in T2DM with DR as compared to without DR contributed to the higher IL-6 level in DR. A significant positive correlation between serum IL-6 and PISA and also between IL-6 and CAL was obtained among the study subjects. DR is a low-grade inflammatory disease and inflammation specifically leukocyte adhesion to the retinal vasculature triggers the disease in a hyperglycemic environment[29]. This study reveals that elevated IL-6 concentrations may contribute to the disease activity in DR. A bidirectional link may exist between DR and PD *via* IL-6 since it might have been involved in the pathophysiology of both conditions.

Lp(a) is an independent risk factor for developing vascular disease. It has the potential to cause vessel damage through lipoprotein oxidation to exert antifibrinolytic and prothrombotic effects. In this study, it is interesting to note that T2DM with DR subjects showed a significantly higher Lp(a) level (approximately 27.76 mg/dL) as compared to the group without DR (11.45 mg/dL). Reports are there in the literature pointing to the relation between serum Lp(a) concentrations and DR[30]. Conflicting reports are also available in the literature. Paige *et al*[31] in 2017 reported an inverse association between Lp(a) concentration and risk of T2DM. A significant positive correlation between serum Lp(a) and PISA and also between Lp(a) and CAL were obtained in the present study.

Lp(a) is susceptible to oxidative modifications, leading to the formation of 'oxidation-specific epitopes' (OSEs). Different OSEs are present on Lp(a) as 'danger-associated molecular patterns', triggering innate immunity[32]. Modified Lp(a) binds and carries MCP-1/CCL2 (pro-inflammatory molecules such as the monocyte chemoattractant protein-1), which induces and maintains vascular inflammation.

Inflammation plays an important role in the relationship between periodontitis and DR. Despite both groups having poor glycemic control and comparable plaque scores, the periodontal parameters were higher in DR as compared to T2DM without DR. Since a bidirectional link exists between periodontitis and DM, the presence of DR may have contributed to the severity of periodontal destruction and periodontitis may have influenced the progression of DR.

One of the limitations of this study was its small sample size. Periodontal parameters like PPD and CAL were measured manually using a William's graduated periodontal probe. More accurate results can be obtained with new-generation computerized probes. In this study it was unfeasible to corroborate the causality and the direction of the relationship between periodontitis and type 2 DR, due to its cross-sectional study design. Large multicentric clinical studies with a proper longitudinal study design and appropriate adjustments for confounders are needed to ascertain whether PD affects the progression of DR and DR contributes to the severity of PD.

CONCLUSION

Despite both groups having poor glycemic control and comparable plaque scores, the periodontal and inflammatory parameters were higher in the DR group compared to T2DM without DR. The presence of DR may have contributed to the severity of periodontal destruction and periodontitis may have influenced the progression of DR. Proper periodontal care can help in improving glycemic control and prevent the progression of DR to some extent. A better understanding of the association between type 2 DR and periodontitis will help create awareness among the public and improve their overall quality of life.

ARTICLE HIGHLIGHTS

Research background

The two-way relationship between periodontitis and type 2 diabetes mellitus (T2DM) is well established. Prolonged hyperglycemia contributes to increased periodontal destruction and severe periodontitis, accentuating diabetic complic-

ations. An inflammatory link exists between diabetic retinopathy (DR) and periodontitis.

Research motivation

Studies regarding this relation and the role of lipoprotein(a) [Lp(a)] and interleukin-6 (IL-6) in these conditions are scarce in the literature. This study assessed the proportion and severity of periodontitis and the correlation between periodontal inflamed surface area (PISA), and clinical attachment loss (CAL) with glycated hemoglobin (HbA1c), serum IL-6 and Lp(a).

Research objectives

(1) To determine and compare the proportion and severity of periodontitis in T2DM subjects with and without DR; (2) To assess the correlation between PISA and HbA1c, serum IL-6, and Lp(a) in T2DM subjects with and without DR; and (3) To assess the correlation between CAL and HbA1c, serum IL-6, and Lp(a) in T2DM subjects with and without DR.

Research methods

The duration of the study was 18 months. In this study, 80 T2DM subjects (40 with DR and 40 without DR) were selected from the diabetic clinic of Department of Internal Medicine, Government Medical College, Calicut. They were divided into two groups based on the presence of DR as follows: Group I- T2DM with DR and Group II- T2DM without DR. Subjects were assessed with a detailed questionnaire regarding their socio-demographic characteristics, medical history, oral hygiene practice, history of diabetes and drug allergy. HbA1c, fasting plasma glucose and postprandial plasma glucose, serum IL-6, and Lp(a) were evaluated. Probing pocket depth, CAL, bleeding on probing, oral hygiene index-simplified, PISA and periodontal disease severity were determined. Diagnosis of DR was done by dilated fundoscopy.

Research results

The proportion of periodontitis in T2DM with DR and in T2DM without DR was 47.5% and 27.5% respectively. Severity of periodontitis, CAL, PISA, serum IL-6 and Lp(a) were higher in T2DM with DR group compared to T2DM without DR group. HbA1c was positively correlated with CAL ($r = 0.351$, $P = 0.001$), and PISA ($r = 0.393$, $P \leq 0.001$) in study subjects. A positive correlation was found between PISA and IL6 ($r = 0.651$, $P < 0.0001$); PISA and Lp(a) ($r = 0.59$, $P < 0.001$); CAL and IL6 ($r = 0.527$, $P < 0.0001$) and CAL and Lp(a) ($r = 0.631$, $P < 0.001$) among study subjects.

Research conclusions

The presence of DR may have contributed to the severity of periodontal destruction and periodontitis may have influenced the progression of DR.

Research perspectives

Since a bidirectional link exists between periodontitis and diabetes mellitus, periodontal therapy should be included in the diabetes management. Proper periodontal care can help in improving glycemic control and prevent the progression of DR to some extent. A better understanding of the association between type 2 DR and periodontitis will help to create awareness among the public and to improve their overall quality of life.

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FOOTNOTES

Author contributions: All the authors in this study have contributed significantly and by keeping the latest guidelines by international committee of medical journal editors; Vadakkekuttical RJ has contributed to conception, study design, data analysis, intellectual content, interpretation of data and final approval of the manuscript; Thazhe Poyil NJ has contributed to study design, data analysis, interpretation of data and manuscript drafting and final approval of the manuscript; Radhakrishnan C has contributed to study design, interpretation of data, intellectual content, and final approval of the manuscript; all authors approved the final version to be published.

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Informed consent statement: All the study participants were explained about the study and informed written consent was obtained in the regional language prior to study enrollment of each subject and the documents are kept under safe custody in the department.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest in this study. This study was supported by personnel funds of the authors.

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Association of age at diagnosis of diabetes with subsequent risk of age-related ocular diseases and vision acuity

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Abstract

BACKGROUND

The importance of age on the development of ocular conditions has been reported by numerous studies. Diabetes may have different associations with different stages of ocular conditions, and the duration of diabetes may affect the development of diabetic eye disease. While there is a dose-response relationship bet-

ween the age at diagnosis of diabetes and the risk of cardiovascular disease and mortality, whether the age at diagnosis of diabetes is associated with incident ocular conditions remains to be explored. It is unclear which types of diabetes are more predictive of ocular conditions.

AIM

To examine associations between the age of diabetes diagnosis and the incidence of cataract, glaucoma, age-related macular degeneration (AMD), and vision acuity.

METHODS

Our analysis was using the UK Biobank. The cohort included 8709 diabetic participants and 17418 controls for ocular condition analysis, and 6689 diabetic participants and 13378 controls for vision analysis. Ocular diseases were identified using inpatient records until January 2021. Vision acuity was assessed using a chart.

RESULTS

During a median follow-up of 11.0 years, 3874, 665, and 616 new cases of cataract, glaucoma, and AMD, respectively, were identified. A stronger association between diabetes and incident ocular conditions was observed where diabetes was diagnosed at a younger age. Individuals with type 2 diabetes (T2D) diagnosed at < 45 years [HR (95%CI): 2.71 (1.49-4.93)], 45-49 years [2.57 (1.17-5.65)], 50-54 years [1.85 (1.13-3.04)], or 50-59 years of age [1.53 (1.00-2.34)] had a higher risk of AMD independent of glycated haemoglobin. T2D diagnosed < 45 years [HR (95%CI): 2.18 (1.71-2.79)], 45-49 years [1.54 (1.19-2.01)], 50-54 years [1.60 (1.31-1.96)], or 55-59 years of age [1.21 (1.02-1.43)] was associated with an increased cataract risk. T2D diagnosed < 45 years of age only was associated with an increased risk of glaucoma [HR (95%CI): 1.76 (1.00-3.12)]. HRs (95%CIs) for AMD, cataract, and glaucoma associated with type 1 diabetes (T1D) were 4.12 (1.99-8.53), 2.95 (2.17-4.02), and 2.40 (1.09-5.31), respectively. In multivariable-adjusted analysis, individuals with T2D diagnosed < 45 years of age [β 95%CI: 0.025 (0.009,0.040)] had a larger increase in LogMAR. The β (95%CI) for LogMAR associated with T1D was 0.044 (0.014, 0.073).

CONCLUSION

The younger age at the diagnosis of diabetes is associated with a larger relative risk of incident ocular diseases and greater vision loss.

Key Words: Diabetes; Age at diagnosis; Cataract; Glaucoma; Age-related macular disease; Vision acuity

Core Tip: This is the first prospective cohort study to examine the association of age at the diagnosis of diabetes with main ocular conditions. Our findings suggest the age at the diagnosis of diabetes plays an important role in the association between diabetes and incident cataract, glaucoma, and age-related macular disease as well as vision. A younger age at the diagnosis of diabetes was associated with larger excessive relative risk for ocular conditions and larger vision loss. Type 1 diabetes appears to have potentially more harmful effects.

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INTRODUCTION

Although the age-standardised prevalence of avoidable vision impairment did not change, the global number of cases increased substantially due to the increasing aging population[1]. Cataract, glaucoma, and age-related macular degeneration (AMD) are the first, second, and fourth leading global causes of blindness in individuals aged 50 years and older, which accounted for 15.2 million, 3.6 million and 1.8 million cases, respectively[1,2]. Therefore, it is imperative to identify the important determinants for these ocular conditions.

Previous evidence has highlighted the importance of diabetes in the development of ocular conditions[3,4]. Diabetes has been linked to numerous ocular conditions, including cataract[5], glaucoma[6], and AMD[7]. The United Kingdom Million Women Study, involving 1312051 postmenopausal women, demonstrated that diabetes was an important risk factor for cataract surgery[8]. In contrast, evidence suggests diabetes is not among the leading predictors for glaucoma[9,10], and other studies did not find a significant association between diabetes and glaucoma[11]. Previous studies have been inconsistent regarding the association of diabetes with AMD[7]. Several studies have demonstrated a positive relationship between diabetes and AMD[12,13], but more studies did not find a significant association[14-18].

The importance of age on the development of ocular conditions has been reported by numerous studies[5,7,9,10]. Diabetes may have different associations with different stages of ocular conditions[19], and the duration of diabetes may affect the development of diabetic eye disease[3]. While there is a dose-response relationship between the age at diagnosis of diabetes and the risk of cardiovascular disease and mortality[20,21], whether the age at diagnosis of diabetes is associated with incident ocular conditions remains to be explored. It is unclear which types of diabetes are more predictive of ocular conditions.

It is important to identify the life stage at which a diagnosis of diabetes is associated with the highest risk of major ocular conditions for the prevention or screening of these conditions. Using the UK Biobank, we sought to examine the association between age at the diagnosis of diabetes and the incidence of cataract, glaucoma, and AMD.

MATERIALS AND METHODS

Study population

The UK Biobank is a population-based cohort of more than 500000 participants aged 40-73 years at baseline, recruited between 2006 and 2010 from one of the 22 assessment centres across England, Wales, and Scotland[22]. The design and population of the UK Biobank study have been described in detail elsewhere[22]. The UK Biobank Study's ethical approval had been granted by the National Information Governance Board for Health and Social Care and the NHS North West Multicenter Research Ethics Committee. All participants provided informed consent through electronic signature at the baseline assessment. The data used in this study is available in the UK Biobank database under the application number of 62443.

Population selection for eye disease analysis

Individuals with missing data on self-reported eye health (n = 327891), or those with ocular diseases (n = 26320) at baseline were excluded from the analysis. After the exclusion of individuals with missing values on the age at the diagnosis of diabetes or with other type of diabetes rather than type 1 diabetes (T1D) and type 2 diabetes (T2D, n = 264), 7917 participants with T2D were divided into six groups according to the age at diagnosis: < 45, 45-49, 50-54, 55-59, 60-64, and ≥ 65 years. For each diabetic participant, two controls were randomly selected from those without diabetes at baseline using propensity scores matched by age, gender, ethnicity, education, household income, physical activity, smoking, alcohol consumption, sleep duration, depression, hypertension, heart disease, stroke, body mass index (BMI), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides. This analysis was conducted for each diabetes diagnosis age group. The same method was used to randomly select controls for T1D patients (n = 792, Figure 1).

Population selection for vision impairment analysis

Among 117252 individuals who had their vision acuity assessed, 7274 had diabetes at baseline. After excluding individuals with missing values on diabetes diagnosis age or with other type of diabetes rather than T1D/T2D (n = 585), 6192 with T2D were divided into six groups according to the diagnosis age: < 45, 45-49, 50-54, 55-59, 60-64, and ≥ 65 years. The same method was used to select controls for individuals with T1D (Supplementary Figure 1).

Age at diagnosis of diabetes

First, participants were classified as diabetic if they reported that a doctor had ever told them that they had diabetes (Field code: 2443). For those with a self-reported diagnosis of diabetes, they were asked a follow-up question "What was your age when diabetes was first diagnosed?" Participants with a potentially abnormal age at the diagnosis of diabetes were asked to confirm. Algorithms based on self-reported medical history and medication were used to identify T1D and T2D[23]. Furthermore, the codes for international classification diseases (ICD) were used to define T1D/T2D (Supplementary Table 1). The age at the diagnosis of diabetes (years) was then computed by subtracting the birth date from the initial diagnosed date divided by 365.25.

Ascertainment of ocular conditions

Individuals were classified as having AMD (Field code: 1528), cataract (1278), or glaucoma (1277) if they reported a diagnosis of the corresponding conditions. Cases of ocular conditions were also identified using hospital inpatient records based on ICD codes (Supplementary Table 1). Furthermore, we used surgical procedures by OPCS4 to identify cataract events (codes: C71.2 or C75.1)[24]. The onset date of ocular condition was defined as the earliest recorded code date regardless of source. Person-years were calculated from the date of baseline assessment to the date of onset ocular condition, date of death, or the end of follow-up (December 31, 2020 for England and Wales and January 31, 2021 for Scotland), whichever came first.

Vision acuity test

The baseline vision acuity examination was performed among a sub-cohort of the UK Biobank from June 2009 to July 2010. The procedure for the vision acuity test has been described in detail elsewhere[25]. Presenting distance vision acuity was measured at 4 m or at 1 m (if a participant was unable to read) using the logarithm of the minimum angle of resolution (LogMAR) chart on a computer screen. Vision was defined as the presenting vision acuity in the better-seeing eye in the analysis.

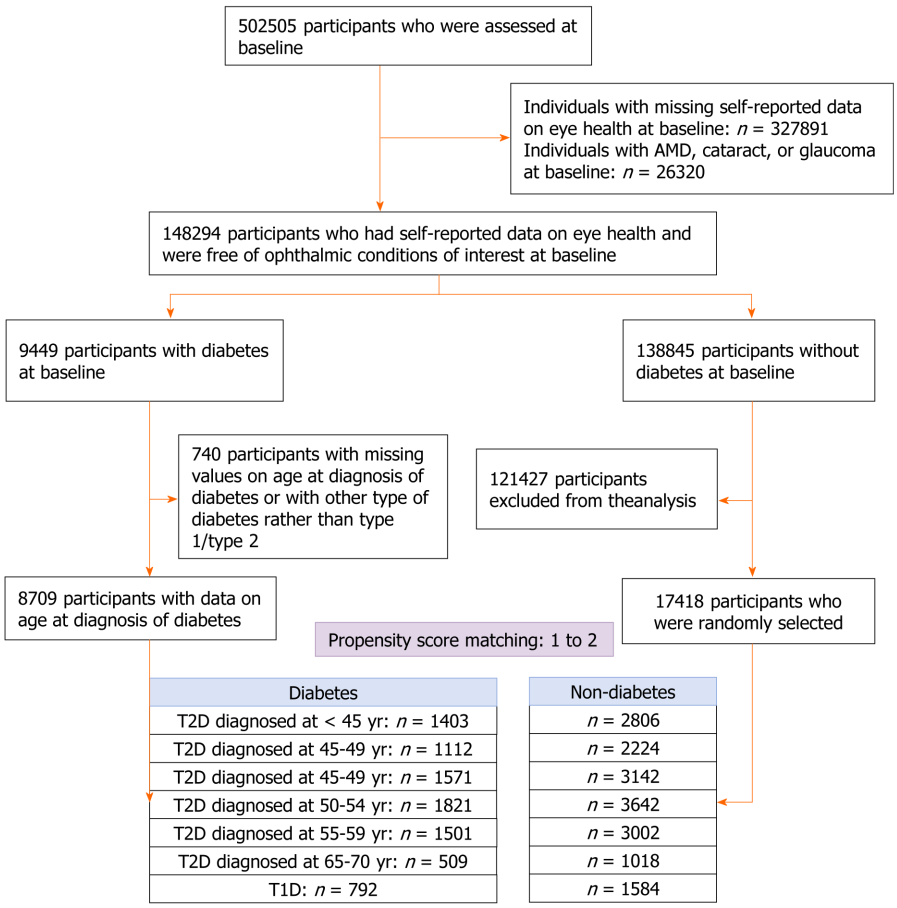

Figure 1 Flowchart for population selection for analysis of ocular conditions from the UK Biobank. Propensity score matching was to select two controls for each diabetic participant. The analysis was conducted for age groups of diabetes diagnosis separately. The median age at diagnosis type 1 was 17 years. Propensity score accounted for age, gender, ethnicity, education, household income, physical activity, smoking, alcohol consumption, sleep duration, depression, hypertension, heart disease, stroke, body mass index, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglyceride. AMD: Age-related macular degeneration; T1D: Type 1 diabetes; T2D: Type 2 diabetes.

Covariates

BMI was calculated as weight in kilograms divided by the square of height in meters. A touchscreen computer was used to collect information, including age, gender, education, income, smoking, alcohol consumption, and sleep duration. Metabolic equivalent-hours/week of physical activity during work and leisure time was estimated using specific questions[26]. Sleep duration was categorized into three groups: < 7, 7-9, and > 9 h[27].

Hypertension, depression, stroke, and heart disease at baseline were defined based on self-reported data. Glycated haemoglobin (HbA1c) was measured using high-performance liquid chromatography on a Bio-Rad Variant II Turbo. Lipids, including total cholesterol, HDL-C, LDL-C, and triglycerides, were measured by direct enzymatic methods (Konelab, Thermo Fisher Scientific, Waltham, Massachusetts).

Statistical analysis

T-test was used to test the difference in continuous variables and Chi-square test in categorical variables between diabetic participants and controls in each diabetes diagnosis age group.

The HR with 95%CIs for incident ocular condition associated with T1D and age at diagnosis of T2D was estimated using Cox proportional hazard regression models. The multivariable analysis included adjustment for matching factors (propensity score) and the full model further incorporated concurrent HbA1c. This analysis was separately conducted for incidence of cataract, cataract surgery, glaucoma, and AMD. The analysis was not performed for types of glaucoma or AMD due to their low incidence.

General linear regression models were used to test the difference in LogMAR between diabetic participants and controls for each diagnosis age group. The multivariable analysis included adjustments for matching factors (propensity score). The association between age at the diagnosis of diabetes and intraocular pressure (IOP) was examined using general linear regression models.

A sensitivity analysis was conducted to examine whether the association between age at the diagnosis of T2D and ocular conditions and vision acuity was independent of duration of diabetes. In this analysis, two controls for each T2D patient were randomly selected using propensity score matching based on the same factors as depicted in Figure 1 and Supplementary Figure 1, without stratification by the age at the diagnosis of diabetes. The age at the diagnosis of T2D, treated as a categorical variable (< 45, 45-49, 50-54, 55-59, 60-64, and ≥ 65 years), was analysed to assess the association

between the age at the diagnosis of diabetes and ocular conditions and vision acuity.

Missing values for categorical variables were assigned as a single category. Missing values for continuous covariates were imputed with the mean.

Data analyses were conducted using SAS 9.4 for Windows (SAS Institute Inc.), and all *P* values were two-sided, with statistical significance set at < 0.05.

RESULTS

Baseline characteristics

For ocular condition analysis, 26127 participants (36.9% females) aged 40-70 (mean ± SD: 59.1 ± 8.2) years old were included in the analysis. Diabetic participants had higher HbA1c, and education levels compared to the controls. No significant difference in other characteristics between the two groups were observed (Table 1). Individuals with T1D had higher HbA1c but did not differ in other characteristics compared to the controls (Supplementary Table 2).

For vision acuity analysis, 20067 participants (37.8% females) aged 40-70 years (mean ±S D: 59.9 ± 7.9), were included. Diabetic participants across all age groups of diabetes diagnosis had higher HbA1c than the controls (Table 2). Individuals with T1D were more likely to have a normal sleep duration and higher HbA1c compared to the controls (Supplementary Table 3).

Incidence of ocular conditions

Over a median follow-up of 11.0 years (interquartile range: 10.7-11.5), 3874 new cases of cataract, 665 new cases of glaucoma, and 616 new cases of AMD were identified.

Age at diagnosis of diabetes and incident AMD, cataract, and glaucoma

As shown in Figure 2, the relative risk for incident AMD associated with diabetes decreased with the increasing age at diagnosis of diabetes. In the multivariable-adjusted analysis, T2D diagnosed at age of < 45 [HR (95%CI): 2.71 (1.49-4.93)], 45-49 [2.57 (1.17-5.65)], 50-54 [1.85 (1.13-3.04)], or 55-59 years [1.53 (1.00-2.34)] was associated with a higher risk of incident AMD. T1D [HR (95%CI): 4.12 (1.99-8.53)] was associated with an increased risk of AMD independent of concurrent HbA1c.

Similarly, the association between diabetes and glaucoma was dependent on the age at diagnosis of diabetes. After adjustment for HbA1c and other covariates, only diabetes diagnosed at age of < 45 years only [HR (95%CI): 1.76 (1.00-3.12)] was associated with an increased risk of glaucoma. The multivariable-adjusted HR (95%CI) for glaucoma associated with T1D was 2.40 (1.09-5.31).

In the multivariable-adjusted model, the HRs (95%CIs) for incident cataract associated with diabetes diagnosed at < 45, 45-49, 50-54, and 55-59 years of age were 2.18 (1.71-2.79), 1.54 (1.19-2.01), 1.60 (1.31-1.96), and 1.21 (1.02-1.43), respectively. T1D was independently associated with an increased risk of incident cataract [2.95 (2.17-4.02)].

As shown in Supplementary Figure 2, T2D diagnosed at < 45, 45-49, 50-54, and 55-59, but not 60-64 or ≥ 65 years of age was associated with an increased risk of cataract surgery, where individuals with T2D diagnosed < 45 years had the highest excess risk of cataract surgery [HR (95%CI): 2.67 (1.88-3.79)]. The multivariable-adjusted HR (95%CI) for cataract surgery associated with T1D was 4.63 (3.10-6.93).

Age at diagnosis of diabetes and vision acuity

After adjustment for covariates and HbA1c, individuals with T2D diagnosed at age of < 45 [β 95%CI: 0.025 (0.009, 0.040)], and 50-54 years [0.016 (0.002, 0.029)] had higher LogMAR compared to the corresponding controls. T1D was associated with a larger LogMAR [0.044 (0.015,0.073), Figure 3].

Age at diagnosis of diabetes and IOP

As shown in Figure 4, individuals with T2D diagnosed at < 45 [β (95%CI): 0.88 (0.59, 1.18) mmHg], 45-49 [0.86 (0.53, 1.18) mmHg], and 50-54 years of age [0.78 (0.52, 1.05) mmHg] had higher IOP compared with the controls. The β (95%CI) for IOP associated with T1D was larger [1.15 (0.73,1.56) mmHg].

Sensitivity analysis

Individuals with diabetes diagnosed at < 50 years of age were younger but had a higher incidence of ocular diseases compared with controls (Supplementary Figure 3). A larger HR was observed for those with diabetes diagnosed at older age. After adjustment for covariates, the association was reversed with diabetes diagnosed at younger age associated with a larger HR. This trend remained consistent after further adjustment for diabetes duration (Figure 5). Individuals with diabetes diagnosed at < 45, 45-49, or 50-54 years of age were younger and had higher LogMAR compared with controls (Supplementary Figure 4). Older age at the diagnosis of diabetes was associated with a larger increase in LogMAR compared with controls. However, after adjustment for covariates, diabetes diagnosed at a younger age was associated with a larger increase in LogMAR (Supplementary Figure 5).

Table 1 Baseline characteristics of participants by diabetes and controls for analysis of ocular conditions

	< 45 yr[1]		45-49 yr[1]		50-54 yr[1]		55-59 yr[1]		60-64 yr[1]		≥ 65 yr[1]	
	Non-diabetes	Diabetes	Non-diabetes	Diabetes	Non-diabetes	Diabetes	Non-diabetes	Diabetes	Non-diabetes	Diabetes	Non-diabetes	Diabetes
Age (yr)	51.9 ± 8.4	51.9 ± 7.9	54.7 ± 8.6	54.7 ± 5.8	58.7 ± 7.8	58.7 ± 4.8	62.2 ± 5.9	62.0 ± 3.5	65.0 ± 3.6	64.9 ± 2.4	67.6 ± 2.7	67.6 ± 1.4
Gender												
Female	1032 (36.8)	506 (36.1)	743 (33.4)	400 (36.0)	1163 (37.0)	600 (38.2)	1312 (36.0)	652 (35.8)	1075 (35.8)	541 (36.0)	402 (39.5)	202 (39.7)
Male	1774 (63.2)	897 (63.9)	1481 (66.6)	712 (64.0)	1979 (63.0)	971 (61.8)	2330 (64.0)	1169 (64.2)	1927 (64.2)	960 (64.0)	616 (60.5)	307 (60.3)
Ethnicity												
Whites	1937 (69.0)	968 (69.0)	1634 (73.5)	812 (73.0)	2522 (80.3)	1255 (79.9)	3225 (88.6)	1600 (87.9)	2715 (90.4)	1352 (90.1)	939 (92.2)	469 (92.1)
Non-whites	785 (28.0)	421 (30.0)	535 (24.1)	296 (26.6)	548 (17.4)	297 (18.9)	367 (10.1)	207 (11.4)	245 (8.2)	141 (9.4)	71 (7.0)	36 (7.1)
Unknown	84 (3.0)	14 (1.0)	55 (2.5)	4 (0.4)	72 (2.3)	19 (1.2)	50 (1.4)	14 (0.8)	42 (1.4)	8 (0.5)	8 (0.8)	4 (0.8)
Education												
0-5 yr	863 (30.8)	379 (27.0)	640 (28.8)	266 (23.9)	839 (26.7)	397 (25.3)	907 (24.9)	436 (23.9)	650 (21.7)	318 (21.2)	206 (20.2)	105 (20.6)
6-12 yr	1270 (45.3)	687 (49.0)	1108 (49.8)	597 (53.7)	1524 (48.5)	776 (49.4)	1631 (44.8)	853 (46.8)	1368 (45.6)	681 (45.4)	419 (41.2)	202 (39.7)
≥ 13 yr	567 (20.2)	292 (20.8)	421 (18.9)	225 (20.2)	706 (22.5)	361 (23.0)	1044 (28.7)	493 (27.1)	927 (30.9)	477 (31.8)	369 (36.2)	192 (37.7)
Missing	106 (3.8)	45 (3.2)	55 (2.5)	24 (2.2)	73 (2.3)	37 (2.4)	60 (1.6)	39 (2.1)	57 (1.9)	25 (1.7)	24 (2.4)	10 (2.0)
Household income (pounds)												
< 18000	680 (24.2)	418 (29.8)	575 (25.9)	320 (28.8)	846 (26.9)	470 (29.9)	1075 (29.5)	550 (30.2)	912 (30.4)	528 (35.2)	372 (36.5)	209 (41.1)
18000-30999	529 (18.9)	285 (20.3)	446 (20.1)	242 (21.8)	696 (22.2)	328 (20.9)	868 (23.8)	445 (24.4)	751 (25.0)	371 (24.7)	267 (26.2)	121 (23.8)
31000-51999	535 (19.1)	244 (17.4)	420 (18.9)	211 (19.0)	576 (18.3)	273 (17.4)	600 (16.5)	297 (16.3)	454 (15.1)	218 (14.5)	110 (10.8)	55 (10.8)
52000-100000	369 (13.2)	152 (10.8)	319 (14.3)	129 (11.6)	356 (11.3)	184 (11.7)	353 (9.7)	171 (9.4)	227 (7.6)	102 (6.8)	52 (5.1)	22 (4.3)
> 100000	101 (3.6)	44 (3.1)	67 (3.0)	25 (2.2)	80 (2.5)	34 (2.2)	99 (2.7)	42 (2.3)	55 (1.8)	17 (1.1)	14 (1.4)	5 (1.0)
Unknown	212 (7.6)	103 (7.3)	128 (5.8)	77 (6.9)	169 (5.4)	121 (7.7)	182 (5.0)	96 (5.3)	178 (5.9)	92 (6.1)	57 (5.6)	32 (6.3)
Not answered	380 (13.5)	157 (11.2)	269 (12.1)	108 (9.7)	419 (13.3)	161 (10.2)	465 (12.8)	220 (12.1)	425 (14.2)	173 (11.5)	146 (14.3)	65 (12.8)
Physical activity (MET-minutes/week)	2287 ± 2162	2264 ± 2201	2281 ± 2153	2223 ± 2176	2205 ± 1959	2197 ± 2142	2350 ± 2204	2366 ± 2231	2475 ± 2230	2376 ± 2188	2553 ± 2113	2523 ± 2360
Alcohol consumption												
Never	365 (13.0)	209 (14.9)	250 (11.2)	142 (12.8)	296 (9.4)	148 (9.4)	272 (7.5)	121 (6.6)	191 (6.4)	92 (6.1)	73 (7.2)	33 (6.5)
Previous	196 (7.0)	125 (8.9)	141 (6.3)	96 (8.6)	201 (6.4)	129 (8.2)	216 (5.9)	130 (7.1)	141 (4.7)	116 (7.7)	61 (6.0)	33 (6.5)

Current	2192 (78.1)	1063 (75.8)	1804 (81.1)	868 (78.1)	2623 (83.5)	1288 (82.0)	3144 (86.3)	1563 (85.8)	2662 (88.7)	1292 (86.1)	881 (86.5)	442 (86.8)
Missing	53 (1.9)	6 (0.4)	29 (1.3)	6 (0.5)	22 (0.7)	6 (0.4)	10 (0.3)	7 (0.4)	8 (0.3)	1 (0.1)	3 (0.3)	1 (0.2)
Smoking												
Never	1549 (55.2)	831 (59.2)	1082 (48.7)	554 (49.8)	1524 (48.5)	741 (47.2)	1605 (44.1)	779 (42.8)	1261 (42.0)	627 (41.8)	406 (39.9)	191 (37.5)
Former	846 (30.1)	366 (26.1)	783 (35.2)	380 (34.2)	1245 (39.6)	639 (40.7)	1621 (44.5)	840 (46.1)	1472 (49.0)	728 (48.5)	511 (50.2)	274 (53.8)
Current	368 (13.1)	193 (13.8)	341 (15.3)	173 (15.6)	355 (11.3)	176 (11.2)	400 (11.0)	188 (10.3)	251 (8.4)	133 (8.9)	99 (9.7)	41 (8.1)
Missing	43 (1.5)	13 (0.9)	18 (0.8)	5 (0.4)	18 (0.6)	15 (1.0)	16 (0.4)	14 (0.8)	18 (0.6)	13 (0.9)	2 (0.2)	3 (0.6)
Sleep duration (h)												
< 7	871 (31.0)	462 (32.9)	685 (30.8)	391 (35.2)	907 (28.9)	475 (30.2)	957 (26.3)	517 (28.4)	705 (23.5)	359 (23.9)	249 (24.5)	124 (24.4)
7-9	1768 (63.0)	845 (60.2)	1436 (64.6)	653 (58.7)	2076 (66.1)	1002 (63.8)	2533 (69.5)	1205 (66.2)	2151 (71.7)	1071 (71.4)	727 (71.4)	358 (70.3)
> 9	107 (3.8)	72 (5.1)	57 (2.6)	52 (4.7)	106 (3.4)	70 (4.5)	116 (3.2)	80 (4.4)	112 (3.7)	56 (3.7)	31 (3.0)	18 (3.5)
Missing	60 (2.1)	24 (1.7)	46 (2.1)	16 (1.4)	53 (1.7)	24 (1.5)	36 (1.0)	19 (1.0)	34 (1.1)	15 (1.0)	11 (1.1)	9 (1.8)
BMI (kg/m^2)	31.4 ± 6.8	31.5 ± 6.5	31.7 ± 6.6	31.9 ± 6.2	31.6 ± 6.4	31.8 ± 5.9	31.1 ± 5.7	31.2 ± 5.4	30.6 ± 5.4	30.8 ± 5.2	30.0 ± 4.8	30.2 ± 4.8
Cholesterol (mmol/L)	4.70 ± 1.02	4.63 ± 1.10	4.64 ± 0.98	4.60 ± 1.09	4.63 ± 0.96	4.57 ± 1.07	4.59 ± 0.97	4.52 ± 1.03	4.65 ± 0.96	4.60 ± 1.04	4.76 ± 1.01	4.66 ± 1.05
HDL-C (mmol/L)	1.22 ± 0.32	1.21 ± 0.32	1.21 ± 0.30	1.21 ± 0.32	1.23 ± 0.31	1.23 ± 0.30	1.23 ± 0.31	1.22 ± 0.30	1.25 ± 0.31	1.25 ± 0.30	1.28 ± 0.31	1.26 ± 0.31
LDL-C (mmol/L)	2.85 ± 0.73	2.83 ± 0.82	2.80 ± 0.71	2.79 ± 0.80	2.77 ± 0.70	2.76 ± 0.78	2.73 ± 0.69	2.72 ± 0.75	2.78 ± 0.70	2.77 ± 0.78	2.86 ± 0.73	2.80 ± 0.77
Triglycerides (mmol/L)	2.03 ± 1.43	2.02 ± 1.22	2.12 ± 1.47	2.10 ± 1.24	2.12 ± 1.43	2.09 ± 1.20	2.15 ± 1.38	2.16 ± 1.23	2.06 ± 1.27	2.08 ± 1.06	2.04 ± 1.20	2.07 ± 1.09
HbA1c (mmol/mol)	36.3 ± 5.4	54.3 ± 17.2[2]	36.7 ± 5.8	52.6 ± 15.5[2]	36.9 ± 5.5	51.4 ± 14.2[2]	37.3 ± 5.3	49.7 ± 12.4[2]	37.3 ± 6.7	47.6 ± 10.8[2]	37.5 ± 4.5	46.5 ± 10.4[2]
Hypertension	1501 (53.5)	764 (54.5)	1307 (58.8)	671 (60.3)	2079 (66.2)	1012 (64.4)	2414 (66.3)	1179 (64.7)	1963 (65.4)	984 (65.6)	643 (63.2)	340 (66.8)
Heart disease	301 (10.7)	147 (10.5)	306 (13.8)	154 (13.8)	450 (14.3)	221 (14.1)	607 (16.7)	293 (16.1)	556 (18.5)	271 (18.1)	183 (18.0)	90 (17.7)
Depression	300 (10.7)	142 (10.1)	167 (7.5)	99 (8.9)	230 (7.3)	109 (6.9)	284 (7.8)	142 (7.8)	160 (5.3)	82 (5.5)	42 (4.1)	22 (4.3)

[1]Age at diagnosis of type 2 diabetes.

[2]Refers to significant difference between diabetes participants and controls. *T*-test was used to test the difference of continuous variables between diabetes participants and controls and Chi-square for categorical variables.

Data are mean ± SD, or *n* (%). BMI: Body mass index; HbA1c: Glycosylated haemoglobin; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; MET: Metabolic equivalent.

DISCUSSION

This large prospective cohort study demonstrated that younger age at diagnosis of diabetes was associated with a larger relative risk for cataract, glaucoma, and AMD independent of concurrent HbA1c levels. Individuals with T2D diagnosed before the age of 45 years were more than twice as likely to develop these ocular conditions, while those with T1D exhibited a more pronounced relative risk. Similarly, T2D diagnosed before the age of 55 years and T1D were associated

Table 2 Baseline characteristics of participants by diabetes and controls for analysis of vision acuity[1]

	< 45 yr		45-49 yr		50-54 yr		55-59 yr		60-64 yr		≥ 65 yr	
	Non-diabetes	Diabetes	Non-diabetes	Diabetes	Non-diabetes	Diabetes	Non-diabetes	Diabetes	Non-diabetes	Diabetes	Non-diabetes	Diabetes
Age (yr)	53.1 ± 8.6	52.8 ± 8.5	54.8 ± 8.5	54.7 ± 5.8	59.0 ± 7.9	58.9 ± 4.8	62.5 ± 5.9	62.3 ± 3.5	65.2 ± 3.6	65.0 ± 2.4	67.5 ± 2.8	67.6 ± 1.3
Gender												
Female	751 (37.6)	379 (37.9)	577 (36.4)	284 (35.8)	937 (39.2)	480 (40.2)	1035 (35.5)	512 (35.1)	962 (36.7)	485 (37.1)	363 (41.4)	177 (40.4)
Male	1249 (62.5)	621 (62.1)	1009 (63.6)	509 (64.2)	1453 (60.8)	715 (59.8)	1879 (64.5)	945 (64.9)	1656 (63.3)	824 (62.9)	513 (58.6)	261 (59.6)
Ethnicity												
Whites	1268 (63.4)	606 (60.6)	1078 (68.0)	518 (65.3)	1793 (75.0)	866 (72.5)	2478 (85.0)	1223 (83.9)	2302 (87.9)	1122 (85.7)	768 (87.7)	379 (86.5)
Non-whites	611 (30.6)	373 (37.3)	454 (28.6)	270 (34.0)	511 (21.4)	306 (25.6)	378 (13.0)	222 (15.2)	267 (10.2)	172 (13.1)	86 (9.8)	54 (12.3)
Unknown	121 (6.1)	21 (2.1)	54 (3.4)	5 (0.6)	86 (3.6)	23 (1.9)	58 (2.0)	12 (0.8)	49 (1.9)	15 (1.1)	22 (2.5)	5 (1.1)
Education												
0-5 yr	585 (29.3)	285 (28.5)	490 (30.9)	215 (27.1)	683 (28.6)	330 (27.6)	755 (25.9)	359 (24.6)	619 (23.6)	306 (23.4)	193 (22.0)	92 (21.0)
6-12 yr	942 (47.1)	487 (48.7)	811 (51.1)	430 (54.2)	1171 (49.0)	589 (49.3)	1359 (46.6)	693 (47.6)	1156 (44.2)	590 (45.1)	375 (42.8)	183 (41.8)
≥ 13 yr	369 (18.5)	192 (19.2)	239 (15.1)	130 (16.4)	462 (19.3)	244 (20.4)	736 (25.3)	370 (25.4)	783 (29.9)	385 (29.4)	287 (32.8)	151 (34.5)
Missing	104 (5.2)	36 (3.6)	46 (2.9)	18 (2.3)	74 (3.1)	32 (2.7)	64 (2.2)	35 (2.4)	60 (2.3)	28 (2.1)	21 (2.4)	12 (2.7)
Household income (pounds)												
< 18000	475 (23.8)	266 (26.6)	359 (22.6)	217 (27.4)	607 (25.4)	330 (27.6)	842 (28.9)	418 (28.7)	796 (30.4)	414 (31.6)	286 (32.6)	165 (37.7)
18000-30999	377 (18.9)	212 (21.2)	346 (21.8)	167 (21.1)	521 (21.8)	244 (20.4)	718 (24.6)	370 (25.4)	673 (25.7)	341 (26.1)	227 (25.9)	106 (24.2)
31000-51999	372 (18.6)	179 (17.9)	313 (19.7)	143 (18.0)	415 (17.4)	205 (17.2)	493 (16.9)	245 (16.8)	402 (15.4)	199 (15.2)	120 (13.7)	55 (12.6)
52000-100000	256 (12.8)	113 (11.3)	234 (14.8)	106 (13.4)	298 (12.5)	151 (12.6)	301 (10.3)	149 (10.2)	220 (8.4)	106 (8.1)	52 (5.9)	23 (5.3)
> 100000	72 (3.6)	34 (3.4)	61 (3.8)	24 (3.0)	77 (3.2)	29 (2.4)	82 (2.8)	36 (2.5)	54 (2.1)	15 (1.1)	14 (1.6)	5 (1.1)
Unknown	132 (6.6)	76 (7.6)	70 (4.4)	56 (7.1)	122 (5.1)	83 (6.9)	117 (4.0)	71 (4.9)	126 (4.8)	71 (5.4)	44 (5.0)	24 (5.5)
Not answered	316 (15.8)	120 (12.0)	203 (12.8)	80 (10.1)	350 (14.6)	153 (12.8)	361 (12.4)	168 (11.5)	347 (13.3)	163 (12.5)	133 (15.2)	60 (13.7)
Physical activity (MET-minutes/week)	2225 ± 2087	2269 ± 2134	2154 ± 2019	2147 ± 2086	2206 ± 2023	2180 ± 2171	2433 ± 2190	2384 ± 2204	2410 ± 2206	2426 ± 2231	2591 ± 2341	2518 ± 2271
Alcohol consumption												
Never	233 (11.7)	156 (15.6)	178 (11.2)	106 (13.4)	205 (8.6)	135 (11.3)	213 (7.3)	107 (7.3)	168 (6.4)	91 (7.0)	60 (6.8)	30 (6.8)
Previous	110 (5.5)	78 (7.8)	96 (6.1)	58 (7.3)	138 (5.8)	85 (7.1)	170 (5.8)	81 (5.6)	131 (5.0)	77 (5.9)	55 (6.3)	34 (7.8)

Current	1595 (79.8)	755 (75.5)	1283 (80.9)	623 (78.6)	2000 (83.7)	966 (80.8)	2509 (86.1)	1260 (86.5)	2294 (87.6)	1133 (86.6)	751 (85.7)	371 (84.7)
Missing	62 (3.1)	11 (1.1)	29 (1.8)	6 (0.8)	47 (2.0)	9 (0.8)	22 (0.8)	9 (0.6)	25 (1.0)	8 (0.6)	10 (1.1)	3 (0.7)
Smoking												
Never	1089 (54.5)	591 (59.1)	778 (49.1)	405 (51.1)	1132 (47.4)	590 (49.4)	1271 (43.6)	648 (44.5)	1144 (43.7)	550 (42.0)	383 (43.7)	182 (41.6)
Former	581 (29.1)	260 (26.0)	563 (35.5)	258 (32.5)	946 (39.6)	458 (38.3)	1308 (44.9)	648 (44.5)	1220 (46.6)	632 (48.3)	410 (46.8)	222 (50.7)
Current	260 (13.0)	130 (13.0)	222 (14.0)	124 (15.6)	273 (11.4)	130 (10.9)	312 (10.7)	145 (10.0)	221 (8.4)	109 (8.3)	70 (8.0)	31 (7.1)
Missing	70 (3.5)	19 (1.9)	23 (1.5)	6 (0.8)	39 (1.6)	17 (1.4)	23 (0.8)	16 (1.1)	33 (1.3)	18 (1.4)	13 (1.5)	3 (0.7)
Sleep duration (h)												
< 7	580 (29.0)	313 (31.3)	474 (29.9)	281 (35.4)	652 (27.3)	347 (29.0)	745 (25.6)	392 (26.9)	594 (22.7)	314 (24.0)	231 (26.4)	98 (22.4)
7-9	1274 (63.7)	625 (62.5)	1025 (64.6)	461 (58.1)	1604 (67.1)	782 (65.4)	2043 (70.1)	982 (67.4)	1880 (71.8)	923 (70.5)	610 (69.6)	320 (73.1)
> 9	64 (3.2)	39 (3.9)	48 (3.0)	37 (4.7)	72 (3.0)	42 (3.5)	90 (3.1)	63 (4.3)	102 (3.9)	55 (4.2)	23 (2.6)	11 (2.5)
Missing	82 (4.1)	23 (2.3)	39 (2.5)	14 (1.8)	62 (2.6)	24 (2.0)	36 (1.2)	20 (1.4)	42 (1.6)	17 (1.3)	12 (1.4)	9 (2.1)
BMI (kg/m^2)	31.3 ± 6.7	31.2 ± 6.7	31.8 ± 6.6	32.0 ± 6.3	31.0 ± 6.1	31.3 ± 5.6	30.8 ± 5.5	30.9 ± 5.1	30.6 ± 5.5	30.6 ± 5.1	29.8 ± 4.9	30.1 ± 4.9
Cholesterol (mmol/L)	4.67 ± 1.01	4.62 ± 1.11	4.63 ± 1.00	4.59 ± 1.08	4.59 ± 0.95	4.58 ± 1.06	4.58 ± 0.95	4.52 ± 1.05	4.68 ± 0.95	4.61 ± 1.04	4.71 ± 0.96	4.68 ± 1.06
HDL-C (mmol/L)	1.24 ± 0.31	1.23 ± 0.33	1.21 ± 0.31	1.22 ± 0.33	1.24 ± 0.30	1.24 ± 0.31	1.24 ± 0.31	1.24 ± 0.30	1.27 ± 0.30	1.26 ± 0.31	1.29 ± 0.32	1.27 ± 0.31
LDL-C (mmol/L)	2.83 ± 0.75	2.81 ± 0.83	2.79 ± 0.72	2.78 ± 0.78	2.75 ± 0.68	2.76 ± 0.78	2.72 ± 0.68	2.70 ± 0.76	2.80 ± 0.69	2.76 ± 0.77	2.83 ± 0.71	2.82 ± 0.78
Triglycerides (mmol/L)	1.88 ± 1.28	1.92 ± 1.15	2.08 ± 1.47	2.07 ± 1.22	2.01 ± 1.37	2.00 ± 1.10	2.11 ± 1.41	2.10 ± 1.21	2.01 ± 1.21	2.02 ± 1.04	1.96 ± 1.19	1.98 ± 1.01
HbA1c (mmol/mol)	36.6 ± 5.3	53.5 ± 16.6[2]	36.6 ± 5.0	52.8 ± 15.6[2]	37.0 ± 4.9	51.2 ± 13.8[2]	37.1 ± 5.3	49.4 ± 12.5[2]	37.3 ± 7.0	47.4 ± 10.9[2]	37.6 ± 10.3	45.5 ± 9.6[2]
Hypertension	1058 (52.9)	528 (52.8)	971 (61.2)	480 (60.5)	1486 (62.2)	746 (62.4)	1883 (64.6)	930 (63.8)	1707 (65.2)	842 (64.3)	547 (62.4)	280 (63.9)
Heart disease	200 (10.0)	99 (9.9)	177 (11.2)	95 (12.0)	314 (13.1)	157 (13.1)	481 (16.5)	230 (15.8)	447 (17.1)	222 (17.0)	158 (18.0)	78 (17.8)
Depression	162 (8.1)	84 (8.4)	129 (8.1)	66 (8.3)	146 (6.1)	70 (5.9)	190 (6.5)	95 (6.5)	127 (4.9)	63 (4.8)	24 (2.7)	12 (2.7)

[1]Age at diagnosis of type 2 diabetes.

[2]Refers to significant difference between diabetes participants and controls. *T*-test was used to test the difference of continuous variables between diabetes participants and controls and Chi-square for categorical variables.

Data are means ± SD, or *n* (%). BMI: Body mass index; HbA1c: Glycosylated haemoglobin; MET: Metabolic equivalent.

with an increased LogMAR. Sensitivity analysis suggests these associations are independent of duration of diabetes.

Diabetes is one of the most important determinants for cataract[8,28,29]. We found that diabetes was associated with an increased risk of incident cataract, and in particular diabetes diagnosed at < 45 years of age had larger excessive risk of cataract. To our knowledge, no previous study has investigated the impact of age at diagnosis of diabetes on the association between diabetes and cataract. However, several studies have shown that the association between diabetes and cataract was stronger among younger than older adults[28-30]. In a cross-sectional analysis, longer duration of

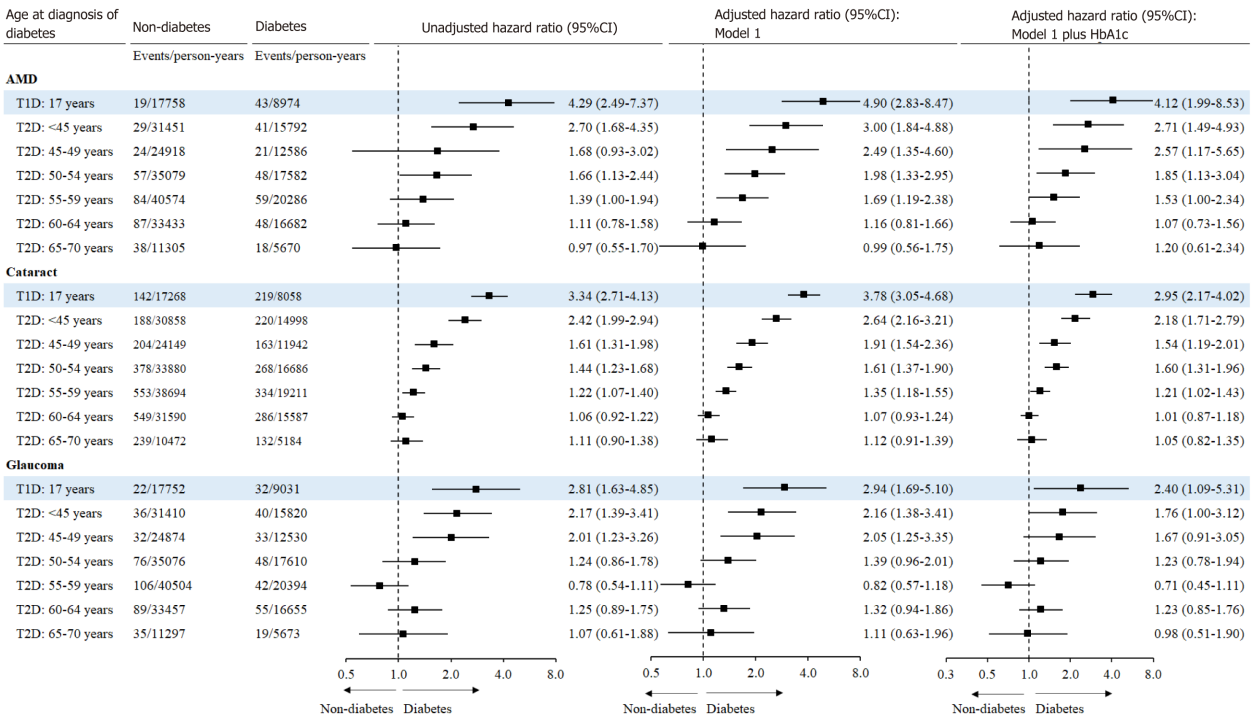

Figure 2 Risk for ocular conditions associated with age at diagnosis of diabetes. Cox proportional hazard regression models were used to examine the association between diabetes and incident ocular condition for each group of diabetes diagnosis age. Model 1 was adjusted for age, gender, ethnicity, income, education, alcohol consumption, physical activity, sleep duration, smoking, body mass index, depression, hypertension, heart disease, stroke, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglyceride. Central squares of each horizontal line represent the hazard ratio for each subgroup. Horizontal lines indicate the range of the 95%CI. The vertical dash lines indicate the hazard ratio of 1.0. AMD: Age-related macular degeneration; T1D: Type 1 diabetes; T2D: Type 2 diabetes; HbA1C: Glycated haemoglobin.

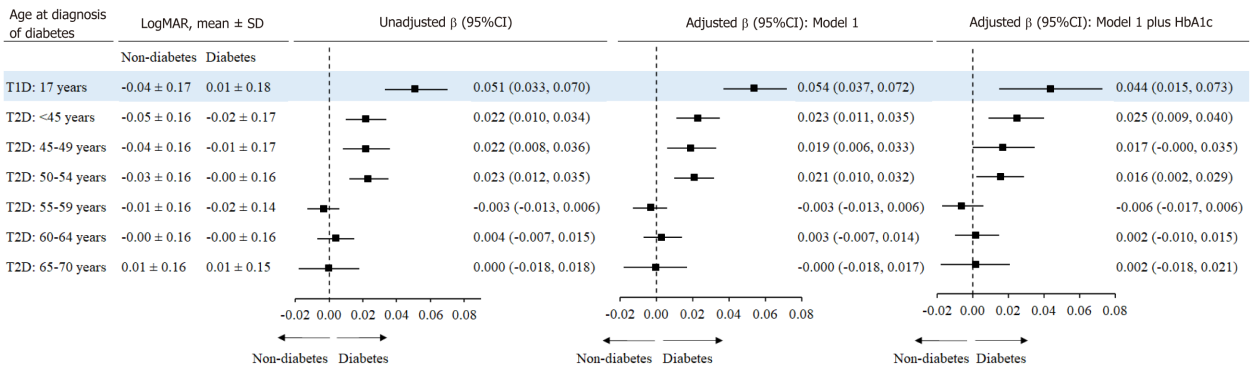

Figure 3 Vision acuity associated with age at diagnosis of diabetes. General linear regression models were used to test the difference in LogMAR between diabetic participants and controls for each group of diabetes diagnosis age. Model 1 was adjusted for age, gender, ethnicity, income, education, alcohol consumption, physical activity, sleep duration, smoking, BMI, depression, hypertension, heart disease, stroke, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglyceride. Central squares of each horizontal line represent the β for each subgroup. Horizontal lines indicate the range of the 95%CI. The vertical dash lines represent the β of 0. T1D: Type 1 diabetes; T2D: Type 2 diabetes; HbA1C: Glycated haemoglobin.

diabetes was associated with a higher prevalence of cataract[28]. These studies provide indirect evidence for the rationale of our findings that younger age at diagnosis of diabetes was associated with a larger excess risk of cataract.

Previous studies have been inconsistent on the association between diabetes and glaucoma. Although a meta-analysis showed that diabetes was associated with a higher risk of glaucoma [relative risk (95%CI): 1.36 (1.25-1.50)], only three out of seven prospective studies included in the meta-analysis found a significant association between diabetes and glaucoma [11]. The lack of significance in some studies may be attributed to a relatively short duration of diabetes[31]. We found individuals with T1D or T2D diagnosed before the age of 45 years but not at 45 years or older had a higher risk of glaucoma. This is consistent with previous studies showing controversial associations between diabetes and glaucoma. It is possible that cumulative exposure to hyperglycemia from an early life may contribute to increased IOP[32], thus elevating the risk of glaucoma. This is supportive by further analysis demonstrating that diabetes diagnosed at a younger age was associated with a larger increase in IOP.

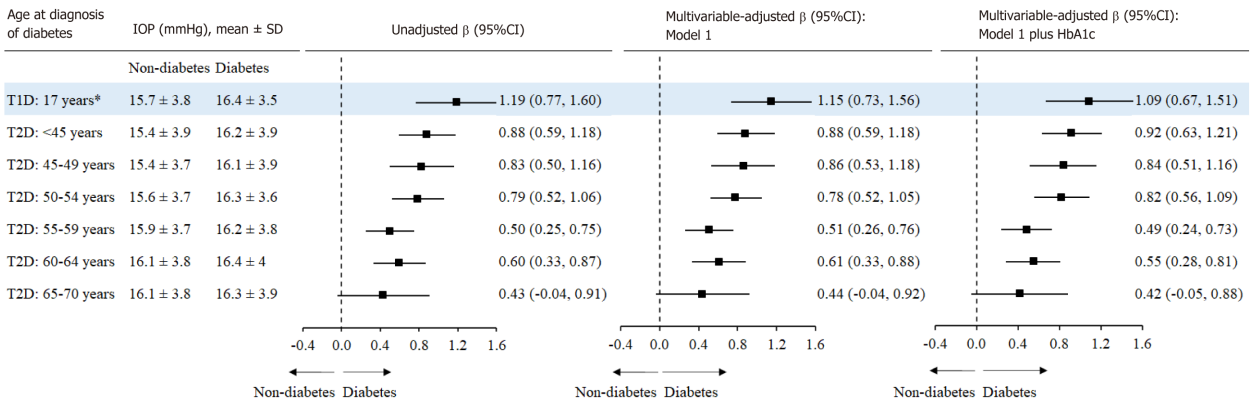

Figure 4 Intraocular pressure associated with age at diagnosis of diabetes. General linear regression models were used to test the difference in intraocular pressure between diabetic participants and controls for each group of diabetes diagnosis age. Model 1 was adjusted for age, gender, ethnicity, income, education, alcohol consumption, physical activity, sleep duration, smoking, body mass index, depression, hypertension, heart disease, stroke, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglyceride. T2D: Type 2 diabetes; IOP: Intraocular pressure.

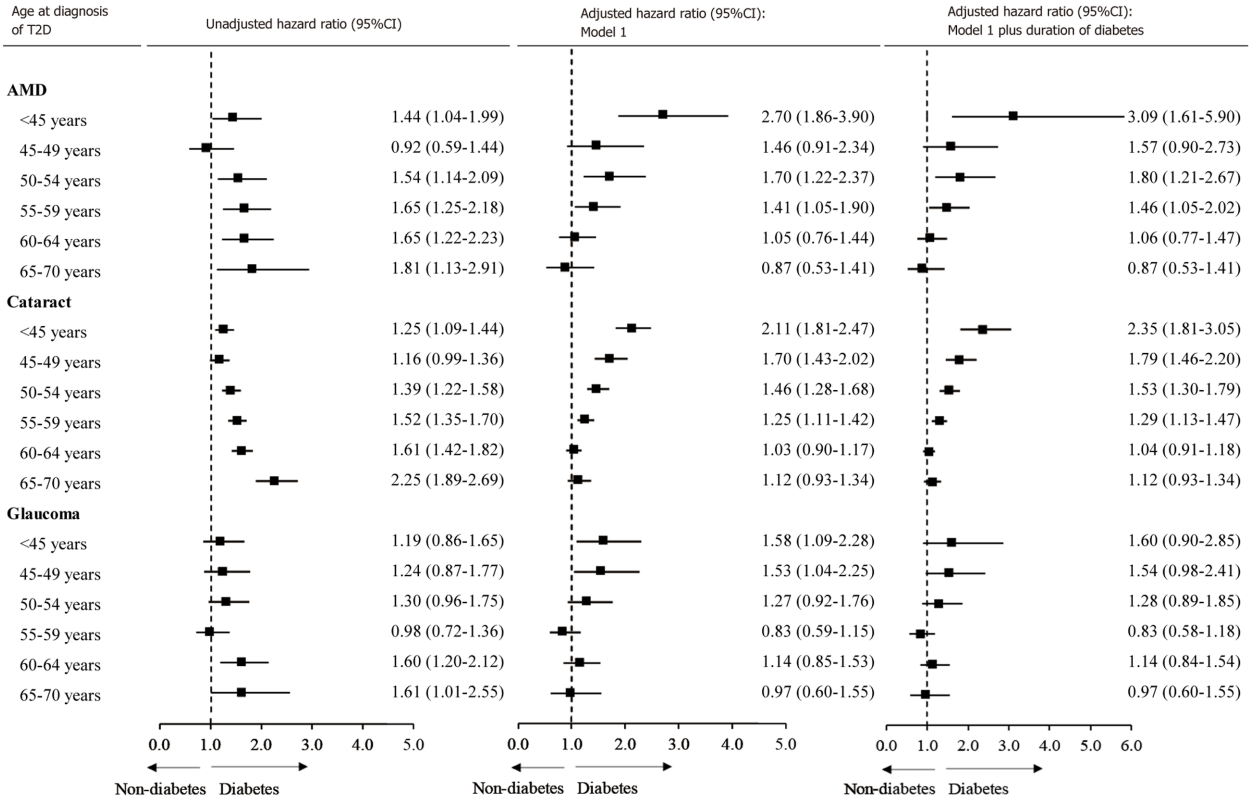

Figure 5 Risk for ocular conditions associated with age at diagnosis of diabetes with the same reference. Sensitivity analysis was conducted to randomly select controls for each individual with type 2 diabetes with all diabetic patients as a whole. Cox proportional hazard regression models were used to estimate hazard ratios for ocular conditions associated with age at diagnosis of diabetes with controls as the reference for each group of diabetes diagnosed age. The multivariable model was adjusted for age, gender, ethnicity, income, education, alcohol consumption, physical activity, sleep duration, smoking, body mass index, depression, hypertension, heart disease, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglyceride, and glycated haemoglobin. Central squares of each horizontal line represent the hazard ratio for each subgroup. Horizontal lines indicate the range of the 95%CI. The vertical dash lines indicate the hazard ratio of 1.0. T2D: Type 2 diabetes; AMD: Age-related macular degeneration.

A meta-analysis showed that diabetes was associated with an increased risk of incident AMD [relative risk (95%CI): 1.05 (1.00–1.11)], although the effect size is small[19]. Among 7 cohort studies in this meta-analysis, only one study reported a significant association[33]. Another prospective study (not included in this meta-analysis) of 71904 patients with diabetes and 270213 patients without diabetes found no significant association between diabetes and incident AMD [34]. A recent prospective study even found that diabetes was associated with a decreased progression of AMD[35]. However, we found that diabetes diagnosed at a younger age but not at an older age was associated with an increased risk of AMD. This finding may offer an explanation for the lack of significant associations reported in most previous studies. Notably, previous studies often combined individuals with diabetes diagnosed at both younger and older ages,

which may introduce a bias towards a null association.

Whether the association between diabetes and incident cataract, glaucoma, or AMD is moderated by the age at diagnosis of diabetes has not been reported in previous studies. However, our study is consistent with a cross-sectional study of 3322 individuals demonstrating that early-onset T2D was associated with a higher prevalence of diabetic retinopathy[36]. Likewise, diabetes diagnosed at a younger age was associated with a larger excess risk of cardiovascular disease and mortality[20]. Our further analysis showed that diabetes diagnosed at < 50 years, but not ≥ 50 years of age was associated with decreased vision. The potential effect of diabetes on the development of ocular conditions and vision loss is independent of HbA1c, highlighting the importance of the age at diabetes diagnosis rather than management of diabetes in this association. T1D was associated with a larger effect size for ocular conditions compared with T2D, even when diagnosed at a younger age. The more potentially harmful effect of T1D may stem from its longer duration of hyperglycaemia and insulin dependency, leading to more extensive damage to blood vessels and nervous system.

The mechanisms undelying the association between a younger age at the diagnosis of diabetes and ocular conditions and vision loss remain largely unknown. A prospective study has shown that T2D developed at a younger age was associated with a higher risk of obesity, worse lipid profiles and higher HbA1c, and a faster deterioration in glycaemic control compared to those with diabetes onset at an older age[37]. These markers have been shown to be important determinants for cataract[38] and glaucoma[31,39] among diabetic patients. This may indicate that early-onset diabetes may represent a more pathogenic condition than late-onset disease for the development of ocular conditions[37]. Furthermore, cumulative exposure to diabetes from early to middle adulthood may exert substantial adverse effects on the development of ocular conditions[40]. The stronger association between early-onset diabetes and ocular conditions may also be attributed to the shared genetics between diabetes and ocular conditions[41-43]. However, the clear pathogenesis of ocular conditions, especially AMD due to diabetes, needs further exploration in research.

To the best of our knowledge, this is the first prospective cohort study to examine the association of age at the diagnosis of diabetes with main ocular conditions. There are several potential limitations in our study. Firstly, some cases of incident ocular conditions may not be captured using inpatient data. Secondly, it is possible that some controls may have developed diabetes during follow-up, which is more likely to bias the associations towards the null. Thirdly, a large proportion of the individuals without eye health data in the UK biobank cohort were excluded from the analysis. This may limit the generalizability of our findings to the whole population.

CONCLUSION

In conclusion, our findings suggest the age at the diagnosis of diabetes plays an important role in the association between diabetes and incident cataract, glaucoma, and AMD as well as vision. A younger age at the diagnosis of diabetes was associated with larger excessive relative risk for ocular conditions and larger vision loss. T1D appears to have potentially more harmful effects.

ARTICLE HIGHLIGHTS

Research background

Diabetes has been linked to numerous ocular conditions, including cataract, glaucomaand age-related macular degeneration (AMD). Several studies have demonstrated a positive relationship between diabetes and AMD, but more studies did not find a significant association. Diabetes may have different associations with different stages of ocular conditions, and the duration of diabetes may affect the development of diabetic eye disease. It is important to identify the life stage at which a diagnosis of diabetes is associated with the highest risk of najor ocular conditions for the prevention or screening of these conditions.

Research motivation

To examine associations between the age of diabetes diagnosis and the incidence of cataract, glaucoma, AMD, and vision acuity. It is important to identify the life stage at which a diagnosis of diabetes is associated with the highest risk of najor ocular conditions for the prevention or screening of these conditions.

Research objectives

To examine associations between the age of diabetes diagnosis and the incidence of cataract, glaucoma, AMD, and vision acuity. A stronger association between diabetes and incident ocular conditions was observed where diabetes was diagnosed at a younger age. It is important to identify the life stage at which a diagnosis of diabetes is associated with the highest risk of major ocular conditions for the prevention or screening of these conditions, and the clear pathogenesis of ocular conditions, needs further exploration in research.

Research methods

This is the first prospective cohort study to examine the association of age at the diagnosis of diabetes with main ocular conditions. Our analysis was using the UK Biobank. The cohort included 8709 diabetic participants and 17418 controls for ocular condition analysis, and 6689 diabetic participants and 13378 controls for vision analysis. Ocular diseases were

identified using inpatient records until January 2021. Vision acuity was assessed using a chart.

Research results

This large prospective cohort study demonstrated that younger age at diagnosis of diabetes was associated with a larger relative risk for cataract, glaucoma, and AMD independent of concurrent glycated haemoglobin levels. Individuals with type 2 diabetes (T2D) diagnosed before the age of 45 years were more than twice as likely to develop these ocular conditions, while those with type 1 diabetes (T1D) exhibited a more pronounced relative risk. Similarly, T2D diagnosed before the age of 55 years and T1D were associated with an increased LogMAR. However, the clear pathogenesis of ocular conditions, especially AMD due to diabetes, needs further exploration in research.

Research conclusions

Our findings suggest the age at the diagnosis of diabetes plays an important role in the association between diabetes and incident cataract, glaucoma, and AMD as well as vision. A younger age at the diagnosis of diabetes was associated with larger excessive relative risk for ocular conditions and larger vision loss. T1D appears to have potentially more harmful effects.

Research perspectives

Investigated the impact of age at diagnosis of diabetes on the association between diabetes and cataract, glaucoma, AMD, and vision acuity, by the more detailed breakdown of factors. To analyse more about the shared genetics between diabetes and ocular conditions.

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FOOTNOTES

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Author contributions: Shang XW, Yang XH, and He MG conceived the study; Shang XW did the literature search and analysed the data; Ye ST, Shang XW, Huang Y, Zhu S, Zhu ZT, and He MG contributed to key data interpretation; Ye ST and Shang XW wrote the manuscript; Ye ST, Shang XW, Huang Y, Zhu S, Zhu ZT, Zhang XL, Wang W, Tang SL, Ge ZY, Yang XH, and He MG critically revised the manuscript. Ye ST is a clinician who identified the clinical problem, provided clinical background, and facilitated the discussion. Shang XW is a statistician who conducted the analysis. Additionally, Ye ST and Shang XW collaborated on drafting the manuscript. Therefore, Ye ST and Shang XW made equal contributions to this work and are co-first authors. He MG provided the funding and the data source, while Yang XH established the research team for this project. They jointly supervised this work. Therefore, they are both considered co-corresponding authors.

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ORIGINAL ARTICLE

Prospective Study

Associations between remnant cholesterol levels and mortality in patients with diabetes

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Abstract

BACKGROUND

Dyslipidemia is frequently present in patients with diabetes. The associations of remnant cholesterol and mortality remains unclear in patients with diabetes.

AIM

To explore the associations of remnant cholesterol with all-cause and cardiovascular mortality in patients with diabetes.

METHODS

This prospective cohort study included 4740 patients with diabetes who participated in the National Health and Nutrition Examination Survey from 1999 through 2018. Remnant cholesterol was used as the exposure variable, and all-cause and cardiovascular mortality were considered outcome events. Outcome data were obtained from the National Death Index, and all participants were followed from the interview date until death or December 31, 2019. Multivariate proportional Cox regression models were used to explore the associations between exposure and outcomes, in which remnant cholesterol was modeled as both a categorical and a continuous variable. Restricted cubic splines (RCSs) were calculated to assess the nonlinearity of associations. Subgroup (stratified by sex, age, body mass index, and duration of diabetes) and a series of sensitivity analyses were performed to evaluate the robustness of the associations.

RESULTS

During a median follow-up duration of 83 months, 1370 all-cause deaths and 389 cardiovascular deaths were documented. Patients with remnant cholesterol levels in the third quartile had a reduced risk of all-cause mortality [hazard ratio (HR) 95% confidence interval (CI): 0.66 (0.52-0.85)]; however, when remnant cholesterol was modeled as a continuous variable, it was associated with increased risks of all-cause [HR (95%CI): 1.12 (1.02-1.21) per SD] and cardiovascular [HR (95%CI): 1.16 (1.01-1.32), per SD] mortality. The RCS demonstrated nonlinear associations of remnant cholesterol with all-cause and cardiovascular mortality. Subgroup and sensitivity analyses did not reveal significant differences from the above results.

CONCLUSION

In patients with diabetes, higher remnant cholesterol was associated with increased risks of all-cause and cardiovascular mortality, and diabetes patients with slightly higher remnant cholesterol (0.68-1.04 mmol/L) had a lower risk of all-cause mortality.

Key Words: Diabetes; Remnant cholesterol; Mortality; Cardiovascular; National Health and Nutrition Examination Survey

Core Tip: This cohort study of 4740 patients with diabetes from the National Health and Nutrition Examination Survey was aimed at evaluating the associations of remnant cholesterol with all-cause and cardiovascular mortality. Diabetes patients with remnant cholesterol levels in the third quartile (0.68-1.04 mmol/L) had a lower risk of all-cause mortality than did nondiabetic patients with remnant cholesterol levels in the other quartiles, and the associations of remnant cholesterol with all-cause and cardiovascular mortality were U-shaped. A per standard deviation increase in remnant cholesterol was associated with a greater risk of all-cause and cardiovascular mortality. A focus should be placed on the level of remnant cholesterol in patients with diabetes.

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INTRODUCTION

The prevalence of diabetes is estimated to be 10.9% by 2030[1]. The prevalence of cardiovascular disease is two to four times greater in patients with diabetes than in patients without diabetes[2]. Dyslipidemia is also frequently present in patients with diabetes as a result of changes in lipoprotein levels triggered by insulin dysfunction and hyperglycemia[3]. Among lipid profile, low-density lipoprotein cholesterol (LDL-C) is highly focused in patients with diabetes[4]. Therefore, statins are widely used in clinical practice for patients with diabetes, irrespective of the presence of complications[5-7]. However, the incidence of major adverse cardiovascular events remains high with the use of current LDL-C lowering strategies[4]. Therefore, there is an evidence gap between existing drug therapies and the prevention of adverse events in patients with diabetes, necessitating a focus on lipoproteins other than LDL-C.

Remnant cholesterol, the remaining cholesterol that is not LDL-C or high-density lipoprotein cholesterol (HDL-C), has been found to be associated with a higher risk of peripheral artery disease, ischemic stroke, *etc.*, and the relationship persisted when controlling for other risk factors, including hypertension and high LDL-C[8-10]. Remnant cholesterol and triglycerides are both carried in lipoproteins that are enriched in triglycerides. Moreover, triglyceride-rich lipoproteins can accumulate in the arterial intima, which may further accelerate the progression of atherosclerosis and increase the risk of cardiovascular events[11]. Thus, in clinical practice, remnant cholesterol should be considered a potential predictor of atherosclerosis and cardiovascular events for individuals with diabetes, and it is essential to assess the impact of remnant cholesterol on mortality among those patients.

Therefore, in this study, we aimed to explore the associations of remnant cholesterol with all-cause and cardiovascular mortality in patients with diabetes using data from the population-based National Health and Nutrition Examination Survey (NHANES).

MATERIALS AND METHODS

Participants

This study included individuals who participated in the NHANES between 1999 and 2018. We included all adults aged ≥ 18 years with diabetes and complete data on total cholesterol (TC), LDL-C, and HDL-C in mmol/L, resulting in a cohort

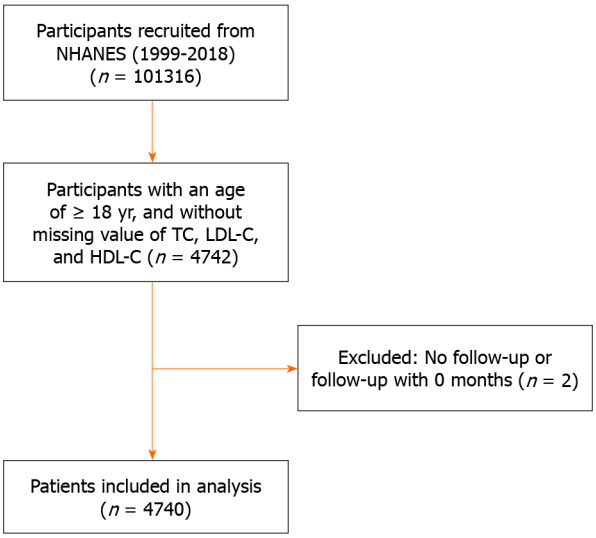

Figure 1 Trial flow chart. TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; NHANES: National Health and Nutrition Examination Survey.

of 4742 patients. We also excluded patients without a follow-up time (or 0 months) (n = 2). Therefore, 4740 patients with diabetes were ultimately included in this study (Figure 1). All procedures were performed in accordance with the Declaration of Helsinki. The NHANES study was reviewed and approved by the NCHS Research Ethics Review Board. Written informed consent was obtained from all participants.

Definition of diabetes

Diabetes status was defined on the basis of the following criteria: Diagnosed with diabetes by a physician, using insulin or oral diabetes medications, hemoglobin A_{1c} ≥ 6.5%, plasma fasting glucose ≥ 126 mg/dL (≥ 7.0 mmol/L) (after at least 8 h of fasting) or 2-h blood glucose ≥ 200 mg/dL (≥ 11.1 mmol/L) during an oral glucose tolerance test[12].

Measurement of remnant cholesterol

Blood specimens were collected as part of the NHANES, and lipid profile data, including TC, LDL-C, HDL-C and triglyceride levels, were retrieved from the NHANES website. Using that data, remnant cholesterol was calculated by the following equation: remnant cholesterol (mmol/L) = TC - HDL-C - LDL-C[13].

Ascertainment of mortality

We obtained death data by linking the cohort data with the National Death Index (NDI) through December 31st, 2019. All-cause mortality was defined as death for any reason. Cardiovascular mortality was defined by using the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision codes I00 to I78.

Assessment of covariates

Demographic and lifestyle information was collected *via* standard questionnaires during in-person interviews. The demographic covariates included age (continuous), sex (male and female), ethnicity (Mexican American, other Hispanic, non-Hispanic white, non-Hispanic white, and others), poverty-income ratio (< 1, 1-3, ≥ 3 or unknown), education (less than 9th grade, 9-11th grade, high school graduate, college, college graduate or above or unknown), body mass index (BMI) (continuous), and survey periods (1999-2000, 2001-2002, 2003-2004, 2005-2006, 2007-2008, 2009-2010, 2011-2012, 2013-2014, 2015-2016 or 2017-2018). Lifestyle covariates included self-reported smoking status (every day, some days, not at all, or unknown) and self-reported alcohol consumption (nondrinker, 1-3 drinks per day, ≥ 4 drinks per day or unknown). Clinical covariates included self-reported history of hypertension (yes, no or unknown), hypercholesterolemia (yes, no or unknown), heart failure (yes, no or unknown), coronary heart disease (yes, no or unknown), and cancer (yes, no or unknown).

Statistical analysis

In the present study, all analyses incorporated sample weights, clustering and stratification given the complex sampling design of the NHANES. For normally distributed continuous variables, the data are presented as the mean and SD; for nonnormally distributed continuous variables, the data are presented as the median and interquartile range. Categorical variables are presented as percentages. Differences in age were analyzed using student's t test. Differences in nonnormally distributed continuous variables were analyzed using the Mann-Whitney U test. Differences among categorical variables were analyzed using Pearson's χ^2 test. Hazard ratios (HRs) and 95% confidence intervals (CIs) of remnant cholesterol for all-cause mortality and cardiovascular mortality were calculated by Cox hazards models, adjusting for age, sex, ethnicity, BMI, poverty-income ratio, education, smoking status, alcohol consumption, survey

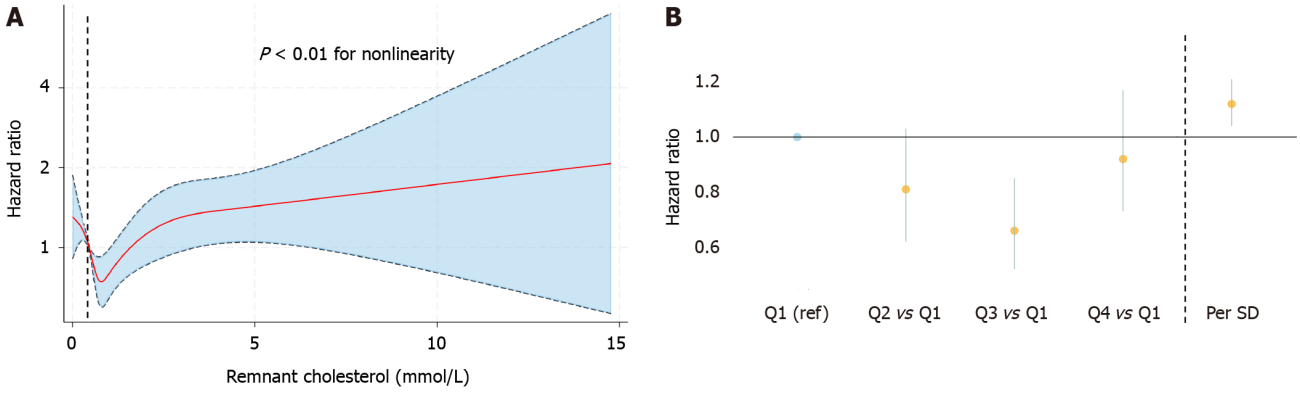

Figure 2 Association of remnant cholesterol with all-cause mortality. A: Multiple-adjusted restricted cubic splines showing hazard ratios (HR) for the risk of incident all-cause mortality associated with remnant cholesterol. Red solid lines represent HRs, and shaded areas represent 95% confidence intervals. Analysis was adjusted for age, gender, ethnicity, body mass index, poverty-income ratio, education, smoking status, alcohol consumption, survey period, hypercholesterolemia, hypertension, heart failure, coronary heart disease, and cancer; B: Multiple-adjusted HRs for remnant cholesterol by quarters and per standard deviation, in association with the risk of all-cause mortality.

period, hypercholesterolemia, hypertension, heart failure, coronary heart disease, and cancer. The group with the lowest quartile of remnant cholesterol was set as the reference group, for which the HR was 1. Additionally, the HR and 95%CI were also calculated for each increase in the SD of remnant cholesterol (treated as a continuous variable). For continuous variables, missing values were imputed by the median value, while for categorical variables, missing data were coded as a separate category "missing".

Stratified analyses were performed by sex (male, female), age (≥ 60 years, < 60 years), BMI (≥ 30 kg/m^2, < 30 kg/m^2), duration of diabetes (≥ 10 years, < 10 years), and ethnicity (Mexican American, non-Hispanic white, non-Hispanic black and others). Furthermore, we conducted the following sensitivity analyses: (1) Excluded patients who died within 1 year of follow-up; (2) further adjusted for serum triglyceride levels; (3) adjusted for lipid-lowering and antihypertensive drugs; and (4) adjusted for cardiovascular mortality. We performed analyses accounting for all-cause death as a competing event with the Fine-Gray competing risks model.

We constructed a restricted cubic spline (RCS) model (knots were selected according to the Akaike information criterion; Supplementary Table 1 to examine the associations of remnant cholesterol with all-cause mortality and cardiovascular mortality (25th percentile as the reference category), adjusting for age, sex, ethnicity, BMI, poverty-income ratio, education, smoking status, alcohol consumption, survey period, hypercholesterolemia, hypertension, heart failure, coronary heart disease, and cancer. A two-sided $P < 0.05$ was set as the threshold for statistical significance. All analyses were performed with Stata 17.0 (StataCorp LLC, College Station, Texas).

RESULTS

Baseline characteristics

A total of 4740 patients with diabetes were included in our analysis. During a median follow-up of 83 months, 1370 all-cause deaths and 389 cardiovascular deaths were observed. Table 1 shows the baseline characteristics stratified by sex. Among them, 2447 patients were male (51.6%). Male patients had a lower BMI and a lower incidence of hypertension. In addition, male patients had a higher fasting serum glucose level. Regarding the lipid profile, we observed higher levels of TC, HDL-C, and LDL-C in female patients with diabetes. However, no significant differences were found in the remnant cholesterol or triglyceride levels between female and male patients.

Remnant cholesterol and all-cause mortality

We observed a nonlinear association between remnant cholesterol levels and all-cause mortality according to the RCS (Figure 2A). When we treated remnant cholesterol as a categorical variable, we found a lower risk in patients in the third quartile of remnant cholesterol than in those in the lowest quartile [Q3: HR (95%CI): 0.66 (0.52-0.85), 0.68-1.04 mmol/L]. Moreover, we found a greater risk of all-cause mortality when remnant cholesterol was modeled as a continuous variable per SD increase [HR (95%CI): 1.12 (1.02-1.21)] (Table 2, Figure 2B).

Remnant cholesterol and cardiovascular mortality

Similarly, the RCS also showed a nonlinear association between remnant cholesterol and cardiovascular mortality (Figure 3A). When remnant cholesterol was modeled as a categorical variable, we also observed a U-shaped association, whereas we did not observe a significantly greater or lower risk in the other three quartiles than in the lowest quartile, despite a favorable trend toward a lower risk in the third quartile [Q3: HR (95%CI): 0.67 (0.42-1.07)]. In addition, we also found a greater risk per SD increase of remnant cholesterol when it was treated as a continuous variable [HR (95%CI): 1.16 (1.01-1.32)] (Table 2, Figure 3B).

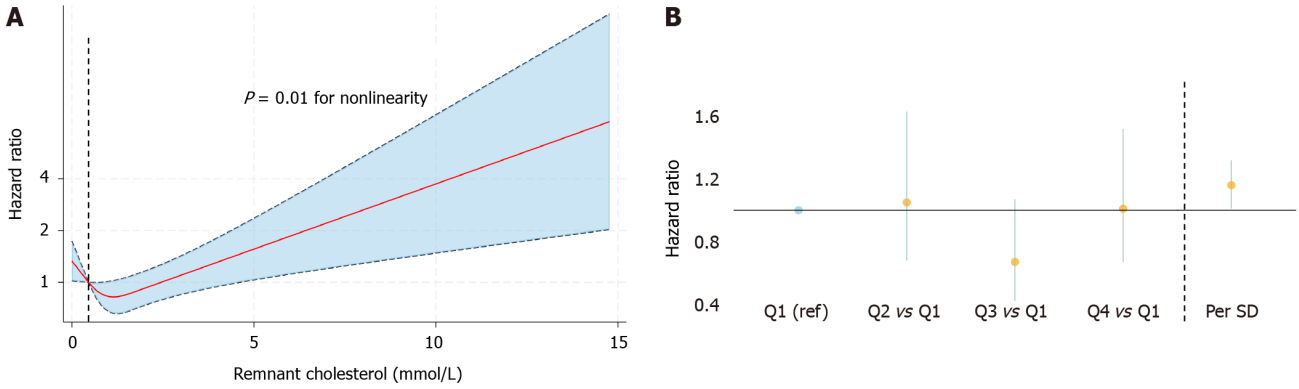

Figure 3 Association of remnant cholesterol with cardiovascular mortality. A: Multiple-adjusted restricted cubic splines showing hazard ratios (HR) for the risk of incident cardiovascular mortality associated with remnant cholesterol. Red solid lines represent HRs, and shaded areas represent 95% confidence intervals. Analysis was adjusted for age, gender, ethnicity, body mass index, poverty-income ratio, education, smoking status, alcohol consumption, survey period, hypercholesterolemia, hypertension, heart failure, coronary heart disease, and cancer; B: Multiple-adjusted HRs for remnant cholesterol by quarters and per standard deviation, in association with the risk of cardiovascular mortality.

Subgroup analyses

We found no interaction between any of the strata and the level of remnant cholesterol (all $P_{interaction}$ > 0.05) regarding the association with all-cause mortality. The corresponding trends of the different strata were also similar. However, the lower risk associated with the third quartile of remnant cholesterol was not observed in patients with diabetes with a BMI > 30 kg/m^2 and a duration of diabetes > 10 years or in Mexican Americans [BMI > 30 kg/m^2, HR (95%CI): 0.77 (0.52-1.12); duration of diabetes over 10 years, HR (95%CI): 0.69 (0.47-1.02); Mexican American ethnicity, HR (95%CI): 0.55 (0.28-1.05)]. Moreover, in non-Hispanic black individuals and individuals of other ethnicities, we observed that there was a trend toward an increase in all-cause mortality risk with elevated remnant cholesterol levels, although no significant increase in risk was associated with any of the individual quartiles of remnant cholesterol.

In addition, there were no significant interactions between any of the strata and remnant cholesterol (all $P_{interaction}$ > 0.05), indicating no evidence of a differential effect of remnant cholesterol on cardiovascular mortality across the different strata. However, among patients in the third quartile of remnant cholesterol, female patients, patients with a BMI < 30 kg/m^2, patients with a duration of diabetes less than 10 years and patients of other ethnicities had a lower risk of cardiovascular mortality [female, HR (95%CI): 0.39 (0.19-0.81); BMI > 30, HR (95%CI): 0.30 (0.15-0.60); duration of diabetes less than 10 years, HR (95%CI): 0.53 (0.29-0.97); other ethnicities, HR (95%CI): 0.12 (0.03-0.45)] (Table 3).

Sensitivity analyses

The results did not change substantially after the following adjustments were implemented: excluding patients who died within 1 year of follow-up; further adjusting for serum triglycerides; adjusting for lipid-lowering and antihypertensive drugs; and treating all-cause mortality as a competing event for cardiovascular mortality. Our results showed that the associations of remnant cholesterol levels with all-cause and cardiovascular mortality were generally robust (Tables 4-7).

DISCUSSION

To our knowledge, this is the first study on the association between remnant cholesterol and mortality in patients with diabetes. In this study, we analyzed data obtained from a population-based database, NHANES, and explored the association of mortality with the NDI. We found that, in patients with diabetes, patients with a remnant cholesterol level in the third-quartile (0.68-1.04 mmol/L) had a lower risk of all-cause mortality. In addition, a similar trend was observed for cardiovascular mortality, but the association between cardiovascular mortality and a remnant cholesterol level in the third quartile was not statistically significant.

Circulating lipoproteins contain both triglycerides and cholesterol. HDL-C and LDL-C primarily transport cholesterol, while other lipoproteins, such as intermediate-density lipoproteins, chylomicrons, and very low-density lipoproteins (VLDLs), not only transport cholesterol but are also enriched with triglycerides[14]. These lipoproteins vary in size, and particles such as VLDLs and chylomicrons may not be able to enter the arterial wall. However, their remnants are able to penetrate into the arterial wall and become trapped. Subsequently, these proteins interact with apoE and apoC-III before being taken up by macrophages[15]. Finally, the accumulation of these remnants accelerates the progression of atherosclerosis[16,17]. A Danish study recruited patients with ischemic heart disease and revealed that those with elevated remnant cholesterol (> 1 mmol/L) had a greater risk of all-cause mortality[18]. In addition to its significant role in exacerbating the progression of atherosclerosis, remnant cholesterol is related to impaired vasodilation and an aggravated inflammatory response[19,20]. Genetic studies have also reported that elevated remnant cholesterol is a causal risk factor for coronary artery disease[21,22]. Another study demonstrated that remnant cholesterol is associated with hepatic steatosis[23]. Moreover, a different study based on NHANES data indicated that patients with hepatic steatosis had an

Table 1 Baseline characteristics

	Male	Female	All participants	*P* value
Sample size	2447	2293	4740	
Age [mean (SD)]	61.8 (13.7)	61.3 (14.3)	61.6 (14.0)	0.09
Body mass index, median (IQR)	29.7 (26.2-33.7)	31.4 (27.4-37.0)	30.5 (26.7-35.4)	< 0.01
Ethnicity, *n* (%)				
Mexican American	466 (19.0)	471 (20.5)	937 (19.8)	< 0.01
Other Hispanic	228 (9.3)	230 (10.0)	458 (9.7)	
Non-Hispanic white	986 (40.3)	790 (34.5)	1776 (37.5)	
Non-Hispanic black	539 (22.0)	611 (26.7)	1150 (24.3)	
Others	228 (9.3)	191 (8.3)	419 (8.8)	
Education, *n* (%)				
Less than 9th grade	547 (22.4)	559 (24.4)	1106 (23.3)	< 0.01
9-11th grade	422 (17.3)	426 (18.6)	848 (17.9)	
High school graduate	631 (25.8)	573 (25.0)	1204 (25.4)	
College	471 (19.3)	491 (21.4)	962 (20.3)	
College graduate or above	365 (14.9)	232 (10.1)	597 (12.6)	
Unknown	11 (0.45)	12 (0.52)	23 (0.48)	
Poverty-income ratio, *n* (%)				
< 1	439 (17.9)	536 (23.4)	975 (20.6)	< 0.01
1-3	1265 (51.7)	1242 (54.2)	2507 (52.9)	
> 3	743 (30.7)	515 (22.5)	1258 (26.6)	
Smoke, *n* (%)				
Every day	299 (12.2)	196 (8.6)	495 (10.4)	< 0.01
Some days	46 (1.9)	43 (1.9)	89 (1.9)	
Not at all	76 (31.7)	414 (18.1)	1190 (25.1)	
Unknown	1326 (54.2)	1640 (71.3)	2966 (62.6)	
Alcohol consumption, *n* (%)				
Non-drinker	358 (14.7)	437 (19.7)	795 (16.8)	< 0.01
1-3 drinks per day	969 (39.6)	745 (32.5)	1714 (36.2)	
≥ 4 drinks per day	378 (15.5)	12 (5.3)	499 (10.1)	
Unknown	742 (30.3)	990 (43.2)	1732 (36.6)	
Hypertension, *n* (%)	1490 (60.9)	1519 (66.3)	3009 (63.5)	< 0.01
Hypercholesterolemia, *n* (%)	1324 (54.1)	1300 (56.7)	2624 (55.4)	0.3
Plasma fasting glucose (mmol/L), median (IQR)	9.8 ± 3.5	8.5 ± 3.5	8.6 ± 3.5	< 0.01
HbA1c, %, median (IQR)	7.2 ± 1.8	7.1 ± 1.7	7.2 ± 1.8	0.21
Remnant cholesterol, median (IQR)	0.68 (0.45-1.07)	0.68 (0.46-1.03)	0.68 (0.46-1.04)	0.53
TC, median (IQR)	4.53 (3.80-5.38)	4.86 (4.11-5.69)	4.68 (3.93-5.53)	< 0.01
HDL-C, median (IQR)	1.09 (0.93-1.32)	1.29 (1.06-1.58)	1.19 (0.98-1.45)	< 0.01
LDL-C, median (IQR)	2.43 (1.71-3.18)	2.59 (1.81-3.34)	2.51 (1.76-3.23)	< 0.01
Triglyceride, median (IQR)	1.48 (1.02-2.21)	1.58 (1.05-2.11)	1.48 (1.04-2.16)	0.97

IQR: Interquartile range; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

Table 2 Association of remnant cholesterol and all-cause and cardiovascular mortality in patients with diabetes

	Hazard ratio (95%CI)					
	Q1	Q2	Q3	Q4	Per SD	*P* value
All-cause mortality						
Model 1	Reference	0.88 (0.68-1.15)	0.65 (0.50-0.84)	0.87 (0.68-1.10)	1.04 (0.97-1.11)	0.01
Model 2	Reference	0.80 (0.62-1.03)	0.67 (0.52-0.86)	0.93 (0.73-1.17)	1.13 (1.04-1.22)	< 0.01
Model 3	Reference	0.80 (0.62-1.02)	0.66 (0.52-0.85)	0.92 (0.72-1.17)	1.13 (1.04-1.22)	< 0.01
Model 4	Reference	0.81 (0.62-1.03)	0.66 (0.52-0.85)	0.92 (0.73-1.17)	1.12 (1.02-1.21)	< 0.01
Cardiovascular mortality						
Model 1	Reference	1.13 (0.72-1.77)	0.60 (0.38-0.96)	0.92 (0.61-1.39)	1.07 (0.95-1.21)	< 0.01
Model 2	Reference	1.01 (0.65-1.55)	0.63 (0.40-0.99)	0.99 (0.66-1.50)	1.19 (1.04-1.37)	< 0.01
Model 3	Reference	1.02 (0.66-1.57)	0.64 (0.41-1.01)	0.98 (0.65-1.48)	1.18 (1.03-1.35)	< 0.01
Model 4	Reference	1.05 (0.68-1.63)	0.67 (0.42-1.07)	1.01 (0.67-1.52)	1.16 (1.01-1.32)	< 0.01

Model 1: No adjustment; Model 2: Adjusted for age, gender, ethnicity, body mass index (BMI), poverty-income ratio, education, survey period; Model 3: Adjusted for age, gender, ethnicity, BMI, poverty-income ratio, education, smoking status, alcohol consumption, survey period; Model 4: Adjusted for age, gender, ethnicity, BMI, poverty-income ratio, education, smoking status, alcohol consumption, survey period, hypercholesterolemia, hypertension, heart failure, coronary heart disease, and cancer.

Table 3 Subgroup analyses on remnant cholesterol and all-cause and cardiovascular mortality

	Hazard ratio (95%CI)				
	Q1	Q2	Q3	Q4	*P* for interaction
All-cause mortality					
Gender					
Male	Reference	0.84 (0.61-1.16)	0.67 (0.48-0.94)	0.96 (0.71-1.32)	0.36
Female	Reference	0.83 (0.57-1.19)	0.64 (0.44-0.93)	0.85 (0.59-1.23)	
Age					
> 60	Reference	0.93 (0.65-1.07)	0.72 (0.55-0.95)	0.89 (0.68-1.17)	0.45
< 60	Reference	0.76 (0.39-1.51)	0.44 (0.24-0.81)	0.91 (0.55-1.51)	
BMI					
> 30	Reference	1.13 (0.78-1.63)	0.77 (0.52-1.12)	1.09 (0.75-1.57)	0.57
< 30	Reference	0.70 (0.51-0.96)	0.66 (0.47-0.93)	0.89 (0.64-1.22)	
Duration of diabetes					
> 10 yr	Reference	0.97 (0.68-1.38)	0.69 (0.47-1.02)	0.78 (0.54-1.12)	0.53
< 10 yr	Reference	0.80 (0.57-1.11)	0.68 (0.49-0.94)	1.02 (0.73-1.41)	
Ethnicities					
Mexican American	Reference	0.59 (0.29-1.20)	0.55 (0.28-1.05)	0.73 (0.39-1.37)	0.85
Non-Hispanic white	Reference	0.85 (0.61-1.19)	0.62 (0.45-0.86)	0.86 (0.63-1.18)	
Non-Hispanic black	Reference	0.91 (0.62-1.33)	0.97 (0.60-1.59)	1.02 (0.62-1.70)	
Others	Reference	0.54 (0.27-1.07)	0.57 (0.28-1.15)	1.37 (0.71-2.63)	
Cardiovascular mortality					
Gender					
Male	Reference	1.40 (0.75-2.61)	1.06 (0.58-1.95)	1.31 (0.76-2.26)	0.17

Female	Reference	0.75 (0.41-1.34)	0.39 (0.19-0.81)	0.76 (0.42-1.37)	
Age					
> 60	Reference	1.09 (0.70-1.71)	0.60 (0.36-1.01)	0.91 (0.58-1.44)	0.3
< 60	Reference	0.78 (0.22-2.81)	1.11 (0.35-3.49)	1.43 (0.46-4.49)	
BMI					
> 30	Reference	1.20 (0.65-2.19)	0.96 (0.50-1.85)	0.87 (0.48-1.58)	0.16
< 30	Reference	1.1 (0.60-2.07)	0.30 (0.15-0.60)	1.55 (0.90-2.66)	
Duration of diabetes					
> 10 yr	Reference	1.03 (0.54-2.00)	0.88 (0.44-1.77)	0.93 (0.47-1.80)	0.4
< 10 yr	Reference	1.14 (0.62-2.03)	0.53 (0.29-0.97)	1.07 (0.62-1.86)	
Ethnicities					
Mexican American	Reference	5.3 (1.44-19.01)	0.72 (0.18-2.91)	4.21 (1.20-14.7)	0.16
Non-Hispanic white	Reference	1.15 (0.65-2.04)	0.70 (0.39-1.26)	0.96 (0.56-1.67)	
Non-Hispanic black	Reference	1.28 (0.69-2.38)	1.17 (0.49-2.81)	1.46 (0.73-2.91)	
Others	Reference	0.35 (0.12-1.06)	0.12 (0.03-0.45)	0.57 (0.18-1.84)	

Model 1: No adjustment; Model 2: Adjusted for age, gender, ethnicity, body mass index (BMI), poverty-income ratio, education, survey period; Model 3: Adjusted for age, gender, ethnicity, BMI, poverty-income ratio, education, smoking status, alcohol consumption, survey period; Model 4: Adjusted for age, gender, ethnicity, BMI, poverty-income ratio, education, smoking status, alcohol consumption, survey period, hypercholesterolemia, hypertension, heart failure, coronary heart disease, and cancer. BMI: Body mass index.

Table 4 Sensitivity analysis of remnant cholesterol and all-cause and cardiovascular mortality after excluding patients who died within 1 year of follow-up

	Hazard ratio (95%CI)					
	Q1	Q2	Q3	Q4	Per SD	*P* value
All-cause mortality						
Model 1	Reference	0.95 (0.72-1.24)	0.70 (0.54-0.92)	0.95 (0.74-1.21)	1.04 (0.97-1.12)	0.04
Model 2	Reference	0.89 (0.69-1.14)	0.72 (0.56-0.93)	0.98 (0.76-1.25)	1.11 (1.02-1.20)	< 0.01
Model 3	Reference	0.88 (0.69-1.13)	0.72 (0.56-0.92)	0.99 (0.77-1.26)	1.11 (1.02-1.21)	< 0.01
Model 4	Reference	0.88 (0.69-1.12)	0.70 (0.54-0.91)	0.97 (0.76-1.25)	1.10 (1.01-1.20)	< 0.01
Cardiovascular mortality						
Model 1	Reference	1.30 (0.81-2.09)	0.65 (0.39-1.07)	0.96 (0.62-1.49)	1.04 (0.91-1.19)	0.05
Model 2	Reference	1.19 (0.76-1.87)	0.66 (0.41-1.07)	0.98 (0.64-1.51)	1.12 (0.97-1.29)	< 0.01
Model 3	Reference	1.20 (0.77-1.88)	0.67 (0.42-1.08)	0.98 (0.64-1.50)	1.11 (0.97-1.28)	< 0.01
Model 4	Reference	1.21 (0.77-1.90)	0.69 (0.43-1.13)	0.98 (0.64-1.52)	1.10 (0.96-1.26)	< 0.01

Model 1: No adjustment; Model 2: Adjusted for age, gender, ethnicity, body mass index (BMI), poverty-income ratio, education, survey period; Model 3: Adjusted for age, gender, ethnicity, BMI, poverty-income ratio, education, smoking status, alcohol consumption, survey period; Model 4: Adjusted for age, gender, ethnicity, BMI, poverty-income ratio, education, smoking status, alcohol consumption, survey period, hypercholesterolemia, hypertension, heart failure, coronary heart disease, and cancer.

increased systemic immune-inflammation index, which is also associated with an increased risk of all-cause mortality[24, 25]. Additionally, the systemic immune-inflammation index was shown to be associated with a higher risk of abdominal aortic calcification, which is also a strong predictor of cardiovascular mortality[26]. In line with the findings of previous studies, our findings similarly demonstrated a greater risk of both all-cause and cardiovascular mortality when analyzing their association with remnant cholesterol levels as a continuous variable.

In patients with diabetes, remnant cholesterol has recently received much attention because of its association with clinical events. For example, in a Chinese cohort study that included 516 individuals diagnosed with type 2 diabetes mellitus, the results indicated that patients with peripheral artery disease exhibited elevated levels of remnant cholesterol,

Table 5 Sensitivity analysis of remnant cholesterol and all-cause and cardiovascular mortality after further adjusting serum triglycerides

	Hazard ratio (95%CI)					
	Q1	Q2	Q3	Q4	Per SD	*P* value
All-cause mortality						
Model 4	Reference	1.30 (0.81-2.09)	0.65 (0.39-1.07)	0.96 (0.62-1.49)	1.04 (0.91-1.19)	< 0.01

Model 4: Adjusted for age, gender, ethnicity, body mass index, poverty-income ratio, education, smoking status, alcohol consumption, survey period, hypercholesterolemia, hypertension, heart failure, coronary heart disease, and cancer.

Table 6 Sensitivity analysis of remnant cholesterol and all-cause and cardiovascular mortality after adjusting for lipid-lowering drug and anti-hypertensive drug

	Hazard ratio (95%CI)					
	Q1	Q2	Q3	Q4	Per SD	*P* value
All-cause mortality						
Model 6	Reference	1.08 (0.70-1.65)	0.65 (0.41-1.03)	0.98 (0.66-1.47)	1.37 (1.13-1.65)	0.05

Model 6: Adjusted for age, gender, ethnicity, body mass index, poverty-income ratio, education, smoking status, alcohol consumption, survey period, hypercholesterolemia, hypertension, heart failure, coronary heart disease, cancer, lipid-lowering drug and anti-hypertensive drug.

Table 7 Sensitivity analysis of remnant cholesterol and cardiovascular mortality after accounting for all-cause death as a competing event by Fine and Gray competing risks model

	Hazard ratio (95%CI)					
	Q1	Q2	Q3	Q4	Per SD	*P* value
Cardiovascular mortality						
Model 4	Reference	1.14 (0.74-1.77)	0.73 (0.46-1.16)	1.03 (0.69-1.55)	1.12 (0.99-1.28)	< 0.01

Model 4: Adjusted for age, gender, ethnicity, body mass index, poverty-income ratio, education, smoking status, alcohol consumption, survey period, hypercholesterolemia, hypertension, heart failure, coronary heart disease, and cancer.

and patients with higher remnant cholesterol (> 0.64 mmol/L) had an increased risk of developing peripheral artery disease[27]. Another study including 4569 white Danish patients with diabetes reported that patients with elevated remnant cholesterol had increased risks of peripheral artery disease, myocardial infarction, ischemic stroke and any atherosclerotic cardiovascular disease[28]. However, despite the established understanding that a higher level of remnant cholesterol increases the risk of clinical events, which was supported in the present study, the exact level of remnant cholesterol associated with mortality in patients with diabetes is still uncertain due to limited study data. A Chinese study indicated that patients with diabetes and diabetic nephropathy who had elevated remnant cholesterol (over 30 mg/dL, 0.77 mmol/L) had a greater risk of cardiovascular mortality than did those with lower remnant cholesterol levels[29]. Cao *et al*[30] reported that individuals with coronary artery disease and diabetes or prediabetes who had elevated remnant cholesterol (> 0.54 mmol/L) had a greater risk of major adverse cardiovascular events. Nonetheless, these studies primarily focused on Asian individuals and were limited by relatively shorter follow-up periods. Studies have demonstrated diverse metabolic statuses in different ethnicities[31,32]. The present study included patients from the NHANES, a nationwide population-based database with diverse ethnicities. Furthermore, this study had a longer follow-up duration, allowing us to draw more reliable conclusions.

Our research included a highly representative population and used a long follow-up period. Furthermore, weights were used in our statistical analysis, leading to credible conclusions. However, several limitations should also be noted. First, mortality was ascertained *via* the NDI, potentially leading to misclassification. However, a prior validation study confirmed the accuracy of the matching method[33]. Second, the estimated remnant cholesterol aligned closely with the measured remnant cholesterol levels at lower LDL-C levels, but a noticeable difference was evident at higher LDL-C levels. However, the measured remnant cholesterol level is not currently provided by the NHANES, which may warrant further investigation. Third, the incidence of cardiovascular mortality was relatively low, which might result in a broad CI and hinder us from examining the true association between remnant cholesterol and cardiovascular mortality. Fourth, dietary inflammation plays an important role in hepatic steatosis and lipid metabolism, especially in patients with

diabetes[34,35]. However, the provision of dietary data was not consistent throughout the entire study period, which limited our ability to arrive at a more comprehensive conclusion.

CONCLUSION

In patients with diabetes, higher remnant cholesterol increased the risk of all-cause and cardiovascular mortality, and diabetes patients with slightly higher remnant cholesterol (0.68-1.04 mmol/L) had a lower risk of all-cause mortality.

ARTICLE HIGHLIGHTS

Research background

Additional research is needed to explore the underlying mechanism of the relationship between remnant cholesterol and mortality.

Research motivation

The optimal remnant cholesterol level for decreasing the risk of all-cause mortality in patients with diabetes was 0.68-1.04 mmol/L. A high level of remnant cholesterol was associated with an increased risk of all-cause and cardiovascular mortality.

Research objectives

The associations of remnant cholesterol with all-cause and cardiovascular mortality were U-shaped. Patients with diabetes in the third quartile of remnant cholesterol (0.68-1.04 mmol/L) had a lower risk of all-cause mortality, and a per standard deviation increase in remnant cholesterol was associated with a higher risk of all-cause and cardiovascular mortality.

Research methods

This cohort study included 4740 patients with diabetes who participated in the National Health and Nutrition Examination Survey from 1999 through 2018. We divided remnant cholesterol into four quartiles, and all participants were followed from the interview date until death or December 31, 2019. Multivariate proportional Cox regression models were used to calculate hazard ratios and 95% confidence intervals. Additionally, a series of subgroup and sensitivity analyses were performed.

Research results

The aim of the present study was to explore the associations of remnant cholesterol with all-cause and cardiovascular mortality in patients with diabetes.

Research conclusions

In current clinical practice, the lipid profile is different between patients with diabetes and nondiabetic patients. However, evidence for the association between remnant cholesterol levels and mortality is lacking.

Research perspectives

Remnant cholesterol is associated with mortality, but the role of remnant cholesterol in patients with diabetes is unclear.

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FOOTNOTES

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Author contributions: Pan D and Xu L performed statistical analysis; Xu L and Zhang LX retrieved raw data from NHANES; Pan D, Shi DZ designed the study. Pan D, Shi DZ and Guo M wrote the manuscript; Xu L and Guo M acquired the fundings supporting the article. The reasons for designating Xu L and Pan D as co-first authors are as follows: First, the research was performed as a collaborative effort, the effort to retrieve raw data is necessary, and statistical analysis is also essential to finish the article. Second, the two authors cooperated together, Pan D designed the study and Xu L made effort to acquire data. Additionally, Pan D wrote the draft, and two

authors managed post-submission matters. We think it is necessary for the article and the two authors contributed with equal importance. Shi DZ and Guo M were co-corresponding authors, the reasons were as follows. Shi DZ inspired the study design and checked the data from NHANES, and confirm the accuracy of the data. Moreover, Shi DZ wrote the draft for the manuscript. Guo M also wrote the draft, and responsible for the revision of the article. In addition, Guo M acquired the fundings and also maintained the raw data. Guo M and Shi DZ are responsible for the data. Hence, we thought Guo M and Shi DZ as co-corresponding authors.

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ORIGINAL ARTICLE

Basic Study

Teneligliptin mitigates diabetic cardiomyopathy by inhibiting activation of the NLRP3 inflammasome

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Abstract

BACKGROUND

Diabetic cardiomyopathy (DCM), which is a complication of diabetes, poses a great threat to public health. Recent studies have confirmed the role of NLRP3 (NOD-like receptor protein 3) activation in DCM development through the inflammatory response. Teneligliptin is an oral hypoglycemic dipeptidyl peptidase-IV inhibitor used to treat diabetes. Teneligliptin has recently been reported to have anti-inflammatory and protective effects on myocardial cells.

AIM

To examine the therapeutic effects of teneligliptin on DCM in diabetic mice.

METHODS

Streptozotocin was administered to induce diabetes in mice, followed by treatment with 30 mg/kg teneligliptin.

RESULTS

Marked increases in cardiomyocyte area and cardiac hypertrophy indicator heart weight/tibia length reductions in fractional shortening, ejection fraction, and heart rate; increases in creatine kinase-MB (CK-MB), aspartate transaminase (AST), and lactate dehydrogenase (LDH) levels; and upregulated NADPH oxidase 4 were observed in diabetic mice, all of which were significantly reversed by teneligliptin. Moreover, NLRP3 inflammasome activation and increased release of interleukin-1β in diabetic mice were inhibited by teneligliptin. Primary mouse cardiomyocytes were treated with high glucose (30 mmol/L) with or without teneligliptin (2.5 or 5 μM) for 24 h. NLRP3 inflammasome activation. Increases in CK-MB, AST, and LDH levels in glucose-stimulated cardiomyocytes were markedly inhibited by teneligliptin, and AMP (p-adenosine 5′-monophosphate)-p-AMPK (activated protein kinase) levels were increased. Furthermore, the beneficial effects of teneligliptin on hyperglycaemia-induced cardiomyocytes were abo-

lished by the AMPK signaling inhibitor compound C.

CONCLUSION
Overall, teneligliptin mitigated DCM by mitigating activation of the NLRP3 inflammasome.

Key Words: Diabetic cardiomyopathy; Teneligliptin; NLRP3; AMPK; Interleukin-1β

Core Tip: Teneligliptin mitigated diabetic cardiomyopathy by mitigating the activation of NLRP3 (NOD-like receptor protein 3) inflammasome. Teneligliptin reversal markedly increased cardiomyocyte area and heart weight/tibia length, reduced fractional shortening, ejection fraction, and heart rate, increased creatine kinase-MB (CK-MB), aspartate transaminase (AST), and lactate dehydrogenase (LDH) levels, and upregulated NADPH oxidase 4 in streptozotocin-induced diabetic mice. Teneligliptin repressed activated NLRP3 inflammasome and increased CK-MB, AST, and LDH levels in glucose-stimulated cardiomyocytes, accompanied by an upregulation of phosphorylated-adenosine 5‘-monophosphate and activated protein kinase.

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INTRODUCTION

With the rapid development of international society and the economy, living standards have improved qualitatively. However, the prevalence of diseases has greatly increased, and diabetes mellitus (DM) is common. According to the International Diabetes Federation, there are 8.75 million people with type 1 diabetes (T1D) worldwide, or 0.11% of the global population. 1.52 million (17.0%) patients were younger than 20 years of age, 5.56 million (64.0%) patients were between 20 and 59 years of age, and 1.67 million (19.9%) patients were 60 years of age or older. In 2022, there will be 530000 newly diagnosed cases of T1D in all age groups, of which 200000 will be under 20 years old[1], posing a threat to public health. As a complication of DM, diabetic cardiomyopathy (DCM) is a condition in which the heart has abnormal myocardial structure and function in the absence of coronary artery disease, severe valvular lesions, or other conventional cardiovascular factors. DCM is characterized by lipid accumulation in the heart, myocardial fibrosis, and an increased probability of myocardial cell death, leading to left ventricular remodeling, hypertrophy, and diastolic dysfunction, which, in turn, contributes to systolic dysfunction[2]. The pathophysiological mechanism of DCM is related to an abnormal glucose supply and lipid metabolism owing to increased oxidative stress (OS), cellular activation regulated by multiple inflammatory pathways, extracellular damage, pathological cardiac remodeling, and diastolic and systolic dysfunction induced by DM[3]. NLRP3 (NOD-like receptor protein 3) is an inflammasome found in both immune and nonimmune cells, such as macrophages, cardiomyocytes, and fibroblasts. NLRP3 is upregulated in multiple diseases, including DCM and atherosclerosis[4]. Activation of the NLRP3 inflammasome has been shown to participate in DCM and the death of cardiomyocytes. In response to a variety of pathological factors, the NLRP3 inflammasome, and the expression of downstream cytokines are triggered, leading to a cascade of inflammatory reactions and causing damage to myocardial cells[5]. Furthermore, NLRP3 activation correlates with reactive oxygen species (ROS) production. Under high glucose conditions, ROS levels are elevated, which is important for NLRP3 inflammasome activation. In addition, ROS activate the NLRP3 inflammasome mainly by forcing cytochrome C to enter the cytoplasm and bind to NLRP3. NLRP3 is inactivated by inhibiting ROS production, which alleviates hyperglycaemia-induced myocardial cell damage [6]. Therefore, NLRP3 is a critical target for treating DCM.

Teneligliptin (Figure 1A) is an oral hypoglycemic dipeptidyl peptidase-IV inhibitor developed by Mitsubishi Pharmaceutical Company in Japan that is mainly used to treat type II diabetes. By suppressing the inactivation of glucagon-like peptide *in vivo* by selectively inhibiting the activity of DPP-IV, teneligliptin promotes the production of insulin by islet cells and reduces the concentration of glucagon, thereby reducing blood glucose. Teneligliptin is well tolerated and has a low incidence of adverse reactions[7,8]. Recently, teneligliptin was shown to have a promising suppressive effect on inflammation[9] and OS[10]. Teneligliptin has been used to treat T2DM patients with renal impairment, including those with end-stage renal disease. Moreover, teneligliptin has cytoprotective effects on myocardial cells[11]. Our study examined the possible therapeutic effectiveness of teneligliptin in DCM in diabetic mice.

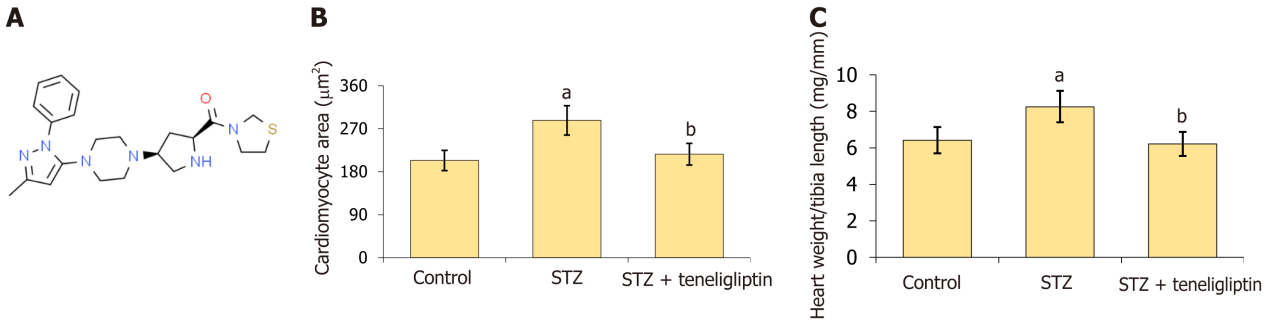

Figure 1 Teneligliptin ameliorated myocardial hypertrophy in streptozotocin-induced diabetic mice. A: Molecular structure of teneligliptin; B: Quantitative analysis of cardiomyocyte area; C: Heart weight/tibia length. [a]*P* < 0.05 *vs* control group; [b]*P* < 0.05 *vs* streptozotocin group, *n* = 8. STZ: Streptozotocin.

MATERIALS AND METHODS

Animal experiments

The streptozotocin (STZ) solution (S0130; Sigma-Aldrich, St Louis, MO, United States) was prepared with citric acid/sodium citrate buffer, and 55 mg/kg STZ was administered to C57BL/6 male mice by intraperitoneal injection for 5 consecutive days. One week later, blood was collected from the tail vein and fasting blood glucose levels were detected by a Roche blood glucose meter. The mouse model of DCM was considered successfully constructed if the fasting blood glucose level was higher than 16.7 mmol/L. The mice were divided into three groups: Vehicle, STZ, and teneligliptin (SML3077; Sigma-Aldrich). In the vehicle and STZ groups, normal mice and diabetic mice received oral doses of normal saline. In the teneligliptin group, diabetic mice were orally administered 30 mg/kg teneligliptin for 4 wk. This study was approved by the Ethics Committee of Jiangxi Provincial People's Hospital.

Measurement of heart function

The mice were anesthetized by an intraperitoneal injection of sodium pentobarbital to maintain a heart rate of approximately 300 beats/min and then placed on a thermostatic heating plate connected to a high-resolution small animal ultrasonic apparatus (VisualSonics, Toronto, ON, Canada). The following parameters were recorded: End-systolic diameter (ESD) and end-systolic volume (ESV), end-diastolic diameter (EDD) and end-diastolic volume (EDV), and heart rate. Fractional shortening was calculated as (EDD-ESD)/EDD × 100%, and the ejection fraction was calculated as (EDV-ESV)/EDV × 100%.

Detection of myocardial injury indicators and interleukin-1β

The levels of creatine kinase-MB (CK-MB), aspartate transaminase (AST), lactate dehydrogenase (LDH), and interleukin (IL)-1β were detected by ELISA (R&D Systems, Minneapolis, MN, United States). The supernatant was collected and added to a 96-well plate and the standards were added. After incubation for 1.5 h, the conjugate solution was added to the wells, after which the plates were incubated for another 1.5 h. Next, tetramethylbenzidine solution (861510; Sigma-Aldrich) was added, followed by a 15 m of incubation. Finally, the stop solution was added to stop the reaction, after which the optical density was detected with a microplate reader (Molecular Devices, San Jose, CA, United States) at 450 nm.

HE staining

The collected myocardium was rinsed with water for 2 h. After being dehydrated with different concentrations of ethanol, the tissues were dehydrated with xylene until transparent, embedded for 1 h and then sliced. After being heated, dewaxed, and hydrated, the sections were immersed in water and subsequently stained with hematoxylin aqueous reagent for 2–3 min. After being differentiated in hydrochloric acid ethanol, the sections were stained with the blue-returning reagent for several seconds. After being stained with eosin for 180 s, images were obtained using an inverted microscope (Nikon, Tokyo, Japan).

Real-time PCR

Total RNA was extracted from tissues or cells with TRIzol reagent, after which the RNA concentration was quantified with an ultraviolet spectrophotometer (Hach, Loveland CO, United States). RNA was transcribed to cDNA using a cDNA synthesis kit (SolelyBio, China). Subsequently, PCR was performed by using an SYBR Premix Ex *Taq*II kit (Takara, Shiga, Japan), and gene expression was determined by using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Tissues or cells were lysed to extract total proteins, followed by quantification using the bicinchoninic acid assay method. The proteins were separated by 12% SDS-PAGE and subsequently transferred to PVDF membranes. After the membranes were blocked, primary antibodies against NADPH oxidase 4 (1:2000, 14347-1-AP; Proteintech, Rosemont, IL, United States), NLRP3 (1:2000, 14347-1-AP; Proteintech), caspase-1 (1:2000, 22915-1-AP; Proteintech), p-AMPK (1:1000, 4186; Cell

Signaling Technology, Danvers, MA, United States), and β-actin (4970; Cell Signaling Technology) were used. Then, secondary antibodies (1:2000, 7074, 7076; Cell Signaling Technology) were added. The bands were visualized with enhanced chemiluminescence solution for quantification.

Primary cardiomyocyte culture

Immature mice (0–2 d of age) were disinfected by immersion in 75% alcohol for 1–2 m. Then, a small incision was made under the sternum with sterilized ophthalmic scissors, after which the heart was collected and was placed in a Petri dish with precooled Hanks solution. The heart was then cut into 1 mm–3 mm pieces with scissors on an ultraclean table, followed by the addition of 7 mL of 0.1% type II collagenase. The rubber plug was blocked, the sealing membrane was added, and cells were then placed in a 37 °C incubator for 10 m until the cells naturally precipitated. The supernatant was discarded, and the digestion was terminated by gently mixing the solution approximately 60 times with a pipettor for 15 m. Then, the cell suspension was transferred to high glucose Dulbecco's modified Eagle's medium. The cell suspension obtained from each digestion was mixed until the tissue block was completely digested. The supernatant was discarded after being centrifuged at 1000 × rpm for 10 m, and an appropriate amount of complete medium was subsequently mixed with a 100 μm nylon screen and inoculated into the culture flask, which was subsequently cultured at 37 °C in a 5% carbon dioxide incubator. After 90 m, the supernatant was mostly purified, and the cardiomyocytes were transferred to another culture flask for further culture.

Statistical analysis

The data are presented as the mean ± SD and were analyzed using one-way analysis of variance with GraphPad Prism software 6.0 (La Jolla, CA, United States). $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Teneligliptin ameliorated myocardial hypertrophy in STZ-induced diabetic mice

WGA staining was used to detect myocardial hypertrophy. Cardiomyocyte area in STZ-treated mice increased from 203.6 μm^2 to 287.5 μm^2 and was markedly reduced to 216.7 μm^2 by teneligliptin (Figure 1B). Moreover, the heart weight/tibia length in STZ-treated mice increased from 6.4 mg/mm to 8.3 mg/mm and was decreased to 6.2 mg/mm by teneligliptin (Figure 1C). Moreover, teneligliptin alleviated myocardial hypertrophy.

Teneligliptin improved heart function in STZ-induced diabetic mice

HE staining (Figure 2A) showed that myocardial cells in the vehicle group had a normal morphology, complete structure, dense and regular arrangement, and uniform distribution of nuclei. However, myocardial cells in diabetic mice were hypertrophic with a fuzzy structure, disordered arrangement, and some fiber breakage. The morphological structures of myocardial cells in the teneligliptin group were neat with relatively regular arrangements and improvements in hypertrophy and fiber breakage. The fractional shortening of diabetic mice decreased from 53.5% to 38.7% and was markedly increased to 49.6% by teneligliptin (Figure 2B). Furthermore, the ejection fractions in the vehicle, STZ, and teneligliptin groups were 87.6%, 73.5%, and 85.5%, respectively (Figure 2C). The heart rates of the diabetic mice decreased from 462.5 beats/min to 458.7 beats/minute and then increased to 468.3 beats/min in response to teneligliptin (Figure 2D). The heart function of diabetic mice was improved by teneligliptin.

Teneligliptin reduced the expression of myocardial injury indicators in STZ-induced diabetic mice

CK-MB levels in diabetic mice increased from 303.5 U/L to 888.8 U/L but were markedly reduced to 578.8 U/L by teneligliptin (Figure 3A). Moreover, AST levels in the vehicle, STZ, and teneligliptin groups were 176.3, 522.6, and 371.8 U/L, respectively (Figure 3B). LDH levels in diabetic mice increased from 262.7 U/L to 697.5 U/L and then decreased to 451.2 U/L in response to teneligliptin (Figure 3C).

Teneligliptin reduced NOX4 levels in diabetic mice

NOX4 is a critical pathological factor in DCM[12]. The increase in NOX4 levels in diabetic mice was markedly inhibited by teneligliptin (Figure 4).

Teneligliptin reduced activation of the NLRP3 inflammasome in the heart in diabetic mice

First, NLRP3 and caspase-1 were notably upregulated in diabetic mice but markedly decreased by teneligliptin (Figure 5A and B). Moreover, IL-1β levels in diabetic mice increased from 2.5 μmol/mg to 3.4 μmol/mg protein and then decreased to 2.8 μmol/mg protein in response to teneligliptin (Figure 5C). The suppressive effect of teneligliptin on the NLRP3 inflammasome in the heart was observed.

Teneligliptin prevented activation of the NLRP3 inflammasome in the heart and injury in cardiomyocytes

Primary mouse cardiomyocytes were extracted and treated with high glucose (30 mmol/L) with or without teneligliptin (2.5, 5 μM) for 24 h. NLRP3 and caspase-1 Levels in cardiomyocytes were markedly increased by 30 mmol/L glucose but were inhibited by 2.5 and 5 μM teneligliptin (Figure 6A). Moreover, IL-1β levels in the control, high glucose, 2.5 μM teneligliptin, and 5 μM teneligliptin groups were 1.5, 2.7, 2.1, and 1.8 μmol/mg protein, respectively (Figure 6B). CK-MB levels increased from 98.8 U/L to 288.5 U/L in 30 mmol/L glucose-treated cardiomyocytes and then decreased to 205.6

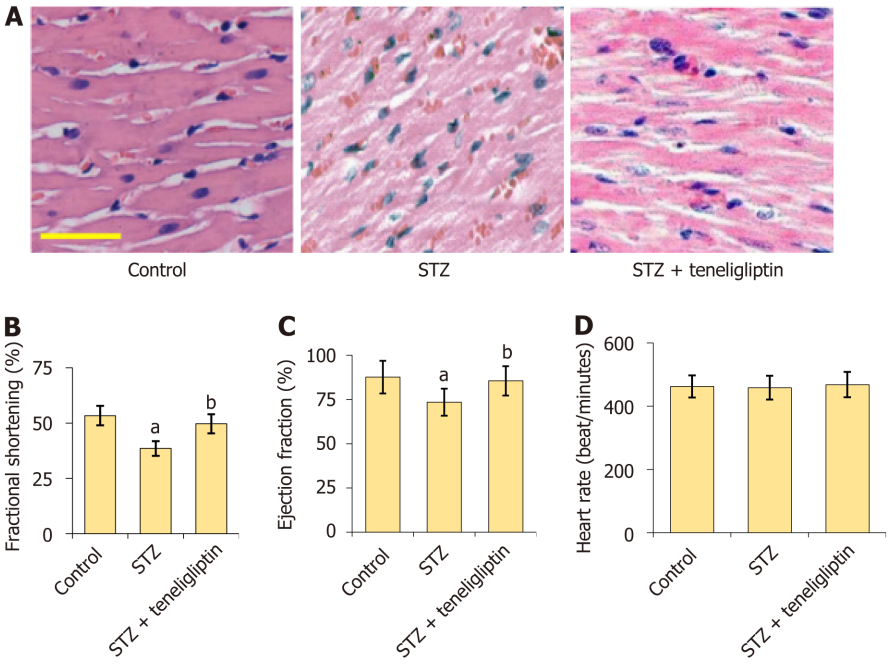

Figure 2 Teneligliptin improved heart function in streptozotocin-induced diabetic mice. A: The results of HE staining, Scale bar = 50 μm; B-D: Mice heart fractional shortening, ejection fraction, and heart rate was measured by echo. [a]*P* < 0.05 *vs* control group; [b]*P* < 0.05 *vs* streptozotocin group, *n* = 8. STZ: Streptozotocin.

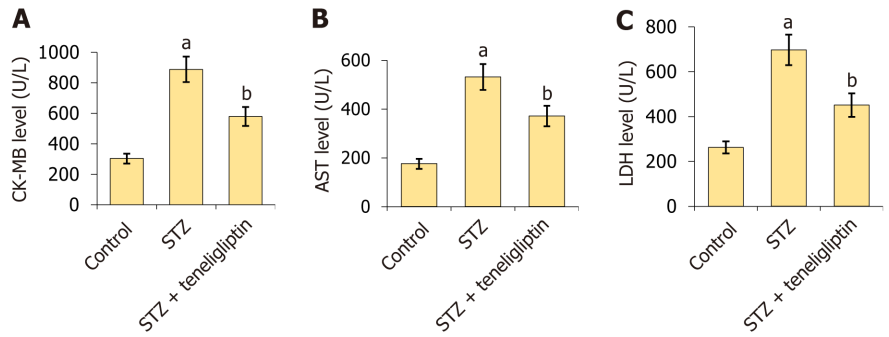

Figure 3 Teneligliptin reduced the expression of the myocardial injury indicators in streptozotocin-induced diabetic mice. A: Creatine kinase-MB level; B: The aspartate transaminase level; C: The lactate dehydrogenase level. [a]*P* < 0.05 *vs* control group; [b]*P* < 0.05 *vs* streptozotocin group, *n* = 8. STZ: Streptozotocin; CK-MB: Creatine kinase-MB; AST: Aspartate transaminase; LDH: Lactate dehydrogenase.

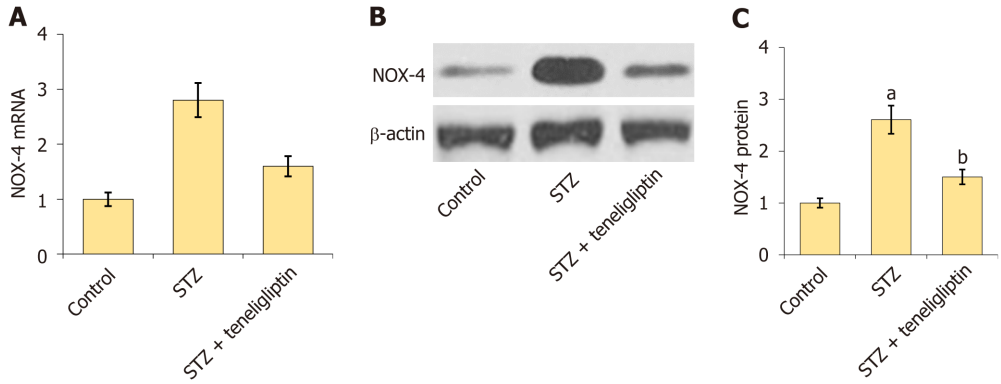

Figure 4 Teneligliptin reduced the expression of NOX4 in diabetic mice. A: mRNA of NOX-4; B: Protein of NOX-4; C: Quantitative analysis of protein expression levels for panel B. [a]*P* < 0.05 *vs* control group; [b]*P* < 0.05 *vs* streptozotocin group, *n* = 8. STZ: Streptozotocin.

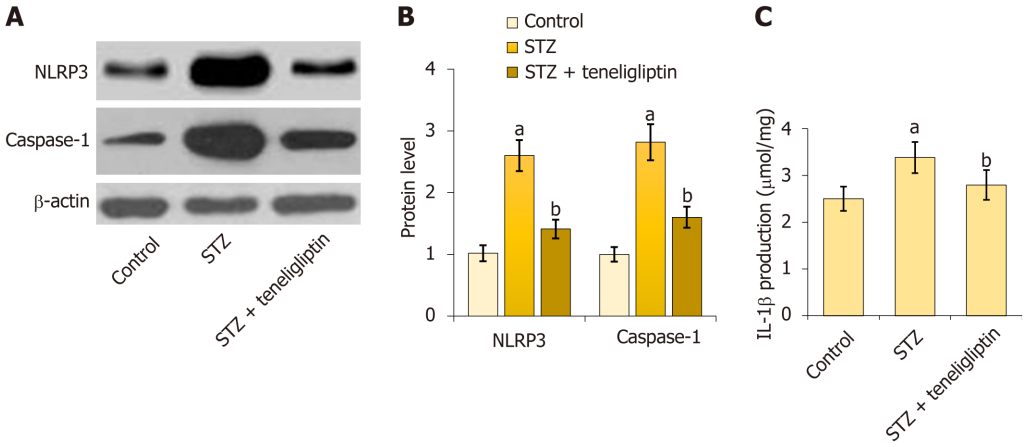

Figure 5 Teneligliptin reduced activation of the cardiac NLRP3 inflammasome in the control and diabetic mice. A: NLRP3 and caspase-1 measured by Western blot assays; B: Quantitative analysis of protein expression levels for panel A; C: Production of interleukin-1β as measured by ELISA. [a]*P* < 0.05 *vs* control group; [b]*P* < 0.05 *vs* streptozotocin group, *n* = 8. STZ: Streptozotocin.

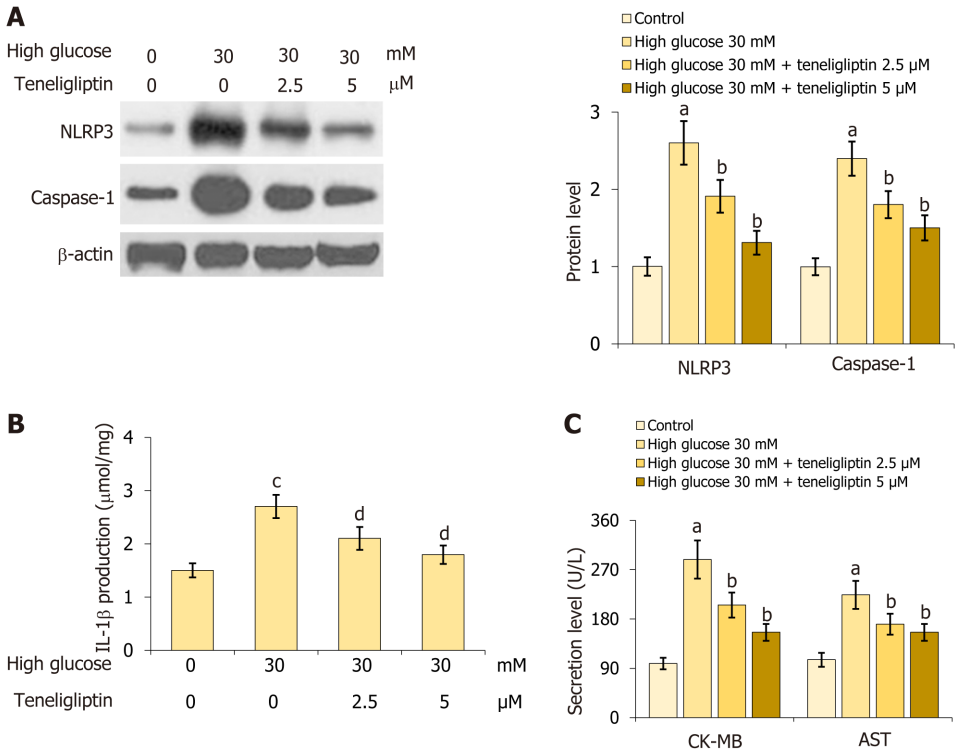

Figure 6 Teneligliptin prevented activation of the cardiac NLRP3 inflammasome and injury in cardiomyocytes. Primary cardiomyocytes were treated with high glucose (30 mmol/L) with or without teneligliptin (2.5 or 5 μM) for 24 h. A: Expression of NLRP3 and caspase-1 were measured by western blot assays; B: Levels of IL-1β as measured by ELISA; C: Levels of creatine kinase-MB and aspartate transaminase level. [a]*P* < 0.05 *vs* control group; [b]*P* < 0.05 *vs* high glucose 30 mmol/L group; [c]*P* < 0.05 *vs* high glucose 0 mmol/L + teneligliptin 0 μM group; [d]*P* < 0.05 *vs* high glucose 30 mmol/L + teneligliptin 0 μM group, *n* = 5. CK-MB: Creatine kinase-MB; AST: Aspartate transaminase; IL: Interleukin.

and 155.7 U/L in response to 2.5 and 5 μM teneligliptin, respectively. Furthermore, AST levels in the control, high glucose, 2.5 μM teneligliptin, and 5 μM teneligliptin groups were 105.6, 223.9, 170.5, and 146.6 U/L, respectively (Figure 6C). The suppressive effect of teneligliptin on the NLRP3 inflammasome in cardiomyocytes was observed.

Treatment with teneligliptin increased the phosphorylation of AMPK in cardiomyocytes under high glucose conditions

AMPK signaling reportedly regulates the NLRP3 inflammasome[13]. The decrease in p-AMPK in cardiomyocytes was reversed by 30 mmol/L glucose and this effect was markedly reversed by 2.5 and 5 μM teneligliptin (Figure 7), suggesting that teneligliptin affected AMPK signaling.

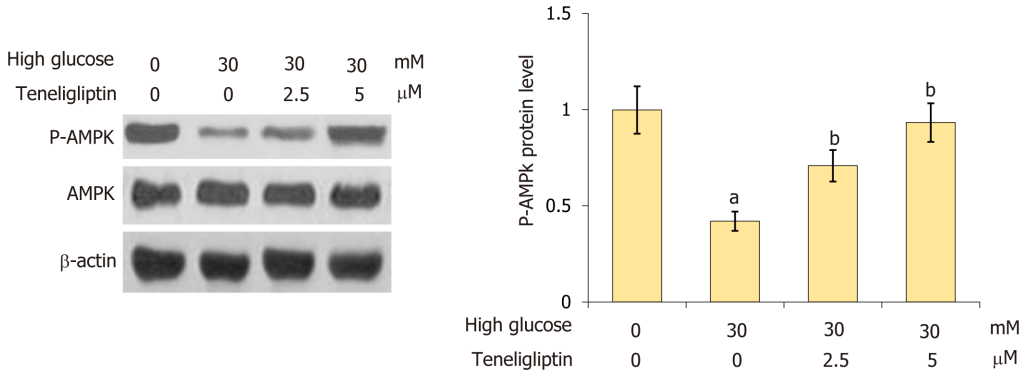

Figure 7 Teneligliptin increased the phosphorylation of AMPK against high glucose in cardiomyocytes. Primary cardiomyocytes were treated with high glucose (30 mmol/L) with or without teneligliptin (2.5 or 5 μM) for 24 h. Levels of p-AMPK were measured by western blot assays. $^{a}P < 0.05$ *vs* control group; $^{b}P < 0.05$ *vs* high glucose 30 mmol/L + teneligliptin 0 μM group, $n = 5$.

Inhibiting AMPK abolished the beneficial effects of teneligliptin on hyperglycaemia-induced cardiomyocytes

For verification, primary mouse cardiomyocytes were treated with high glucose (30 mmol/L) with or without teneligliptin (5 μM) and in the presence or absence of compound C (10 μM) for 24 h. First, the reduction in p-AMPK in 30 mmol/L glucose-treated cardiomyocytes was notably attenuated by teneligliptin, and this effect was reversed by compound C (Figure 8A). Furthermore, the increase in NLRP3 observed in 30 mmol/L glucose-treated cardiomyocytes was strongly reduced by teneligliptin but was elevated by compound C (Figure 8B). Moreover, CK-MB levels increased from 102.5 U/L to 297.6 U/L in 30 mmol/L glucose-treated cardiomyocytes and then greatly decreased to 162.3 U/L in response to teneligliptin. After the administration of compound C, CK-MB levels were reversed to 255.2 U/L. AST levels in the control, high glucose, teneligliptin, and teneligliptin+ compound C groups were 112.3, 235.6, 155.3, and 215.3 U/L, respectively (Figure 8C). The beneficial effects of teneligliptin on hyperglycaemia-induced cardiomyocytes were abolished by AMPK inhibition.

DISCUSSION

Studies have shown that NLRP3 is activated during DCM development through the inflammatory response and that teneligliptin exerts anti-inflammatory and protective effects in myocardial cells. In this study, we established an STZ-induced diabetes model and found that teneligliptin significantly inhibited cardiac hypertrophy and reduced fractional shortening and the ejection fraction induced by diabetes. Additionally, teneligliptin significantly reversed diabetes-induced increases in CK-MB, AST, and LDH. In addition, activation of the NLRP3 inflammasome and the increased release of IL-1β in diabetic mice were inhibited by teneligliptin, which was accompanied by the upregulation of p-AMPK. The same results were obtained in primary mouse cardiomyocytes treated with high glucose.

Teneligliptin is a low-cost oral hypoglycemic dipeptidyl peptidase-IV inhibitor that is used for diabetes treatment. Singh *et al*[14] searched 13 eligible studies containing data on 15720 subjects and showed similar efficacy (or better) and safety of teneligliptin compared with other DPP4Is. The average cost per tablet of teneligliptin (20 mg) was markedly lower than that of sitagliptin, vildagliptin, or other commonly used DPP4Is. Teneligliptin has various effects on both cardiovascular disease and other disorders. Teneligliptin can improve vascular endothelial function by improving flow-mediated vascular dilatation through divergent actions, including changes in circulating endothelial progenitor cells[15]. Teneligliptin-induced vasodilation occurs *via* the activation of protein kinase G, SERCA pumps and protein kinase G channels[16]. A systematic review of 13 randomized controlled trials that enrolled 2853 patients showed that teneligliptin was an effective and safe therapeutic option for patients with T2DM as a monotherapy and add-on therapy[17].

Teneligliptin has recently been reported to have anti-inflammatory and protective effects on myocardial cells, and DPP4 inhibitors have been reported to be associated with mitochondrial metabolism. Notably, teneligliptin enhances SIRT1 protein expression and activity through USP22, a ubiquitin-specific peptidase. Activated SIRT1 prevents hyperglycaemia-induced PRDX3 acetylation by SIRT3, resulting in the inhibition of PRDX3 hyperoxidation and thereby enhancing mitochondrial antioxidant defense[18]. In rat cardiac microvascular endothelial cells, teneligliptin significantly ameliorated the reduction in mitochondrial membrane potential induced by hypoxia/reoxygenation, indicating that teneligliptin could affect mitochondrial function[19]. In 5/6-nephrectomized mice, indoxyl sulfate induced mitochondrial dysfunction by decreasing the expression of PGC-1α and inducing autophagy, as well as decreasing the mitochondrial membrane potential, and teneligliptin reversed this effect[20]. In summary, although teneligliptin has been reported to have anti-inflammatory and protective effects on myocardial cells, future studies should focus on its ability to improve mitochondrial metabolism.

NLRP3 is a NOD-like receptor sensor molecule[21] that belongs to the cytoplasmic NOD family of pattern recognition receptors. After being activated, NLRP3 undergoes self-oligomerization and assembles with ASC and the protease caspase-1 to form the NLRP3 inflammasome. This process results in the production of the active form of the caspase-1 p10/p20 splicer and induces the conversion of the proinflammatory cytokines IL-1β and IL-18 from their immature forms

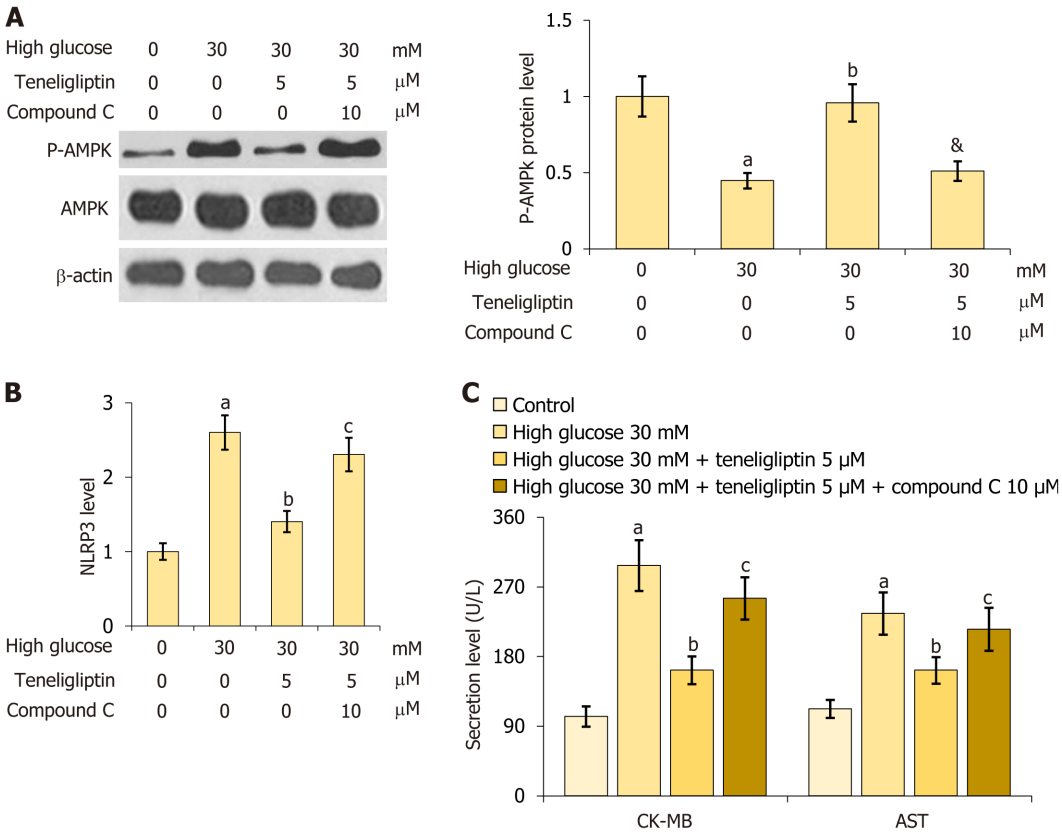

Figure 8 Inhibition of AMPK abolished the beneficial effects of teneligliptin against high glucose in cardiomyocytes. Primary cardiomyocytes were treated with high glucose (30 mmol/L) with or without teneligliptin (5 μM) and compound C (10 μM). A: Levels of p-AMPK; B: The levels of NLRP3; C: Levels of creatine kinase-MB and aspartate transaminase level. [a]*P* < 0.05 *vs* control group; [b]*P* < 0.05 *vs* high glucose 30 mmol/L group; [c]*P* < 0.05 *vs* high glucose 30 mmol/L + teneligliptin 5 μM group, *n* = 5.

to their active forms, inducing an inflammatory response[22]. The NLRP3 inflammasome triggers a form of cell death known as pyroptosis. NLRP3 activation triggers the autocatalytic activation of caspase-1, which drives the cleavage of the gasdermin D protein to produce N'-segment protein fragments that bind to phospholipid proteins on the cell membrane, form holes, and release inflammatory factors. Subsequently, cells continue to expand until the membrane ruptures, which is the process of pyroptosis[23]. NLRP3 inhibition improves insulin sensitivity in obese mice, suggesting that NLRP3 may participate in metabolism-related diseases such as diabetes[24]. In addition, hyperglycemia reportedly activates NLRP3 inflammasome-mediated pyroptosis in myocardial cells, and knocking down NLRP3 in DCM rats reduces the inflammatory response in myocardial tissues, inhibits the occurrence of myocardial pyroptosis and myocardial interstitial fibrosis, and improves cardiac function[25,26]. These findings suggest that the NLRP3 inflammasome is involved in DCM development. In our study, DCM model mice were generated by STZ injection, and the results were verified by assessing myocardial hypertrophy, impaired heart function, and myocardial injury indicator production; these findings were consistent with previous studies[27,28]. Following the administration of teneligliptin, myocardial hypertrophy was alleviated, heart function was improved, and myocardial injury indices were reduced, suggesting that teneligliptin alleviated DCM in mice. Moreover, the NLRP3 inflammasome was activated in both DCM mice and primary mouse cardiomyocytes stimulated with 30 mmol/L glucose, which is consistent with the findings of Song *et al*[29] and Shi *et al*[30]. Following the administration of teneligliptin, NLRP3 inflammasome activity was inhibited in both DCM mice and primary mouse cardiomyocytes stimulated with 30 mmol/L glucose, suggesting that the effect of teneligliptin might be mediated by inhibition of the NLRP3 inflammasome.

This study showed that teneligliptin improved cardiac function in DCM *in vivo* and *in vitro* and exerted its effect through NLRP3. One shortcoming of this study is that the direct effect of NLRP3 was not verified by knocking down or overexpressing NLRP3. In addition, the effect of teneligliptin on NLRP3-induced autophagy will be examined in future studies.

AMPK regulates NLRP3 inflammasome activation through multiple pathways, such as mitochondrial homeostasis, endoplasmic reticulum stress, autophagy, and SIRT1 activation. p-AMPK/AMPK is an important sensor molecule that regulates bioenergy homeostasis and reduces the activation of NF-κB to control the inflammatory response mediated by the NLRP3 inflammasome[31]. A previous study showed that autophagy was induced, mitochondrial ROS production was reduced, and IL-1β secretion was inhibited by mTOR inhibitors[32]. Autophagy is facilitated by AMPK through the activation of ULK or inhibition of the mTOR pathway. Furthermore, NLRP3 inflammasome activity is negatively regulated by autophagy through different mechanisms[33]. In our study, AMPK signaling was inhibited in primary mouse cardiomyocytes stimulated with 30 mmol/L glucose, which is consistent with the findings of Yang *et al*[13]. The sup-

pressive effect of 30 mmol/L glucose on AMPK signaling was reversed by teneligliptin, suggesting that the AMPK pathway mediates the effects of teneligliptin. Moreover, the beneficial effects of teneligliptin on hyperglycaemia-induced cardiomyocytes were abolished by inhibiting AMPK, suggesting that the regulatory effect of teneligliptin on DCM was controlled by AMPK. In the future, the exact mechanism will be identified by treating diabetic mice with teneligliptin plus compound C. This study limitation study should be mentioned. The study did not determine how teneligliptin interfered with AMPK/NLRP3 signaling in cardiomyocytes. It has been reported that NLRP3 plays an important role in cardiomyocyte pyroptosis during DCM. Future studies will further explore the effect of teneligliptin on cardiomyocyte pyroptosis during DCM. LCZ696 is a dual inhibitor of the AT1 receptor and of neprilysin signaling, and it remains unclear whether the effect of LCZ696 is dependent on these two pathways. The role of neprilysin in endothelial cells is not well known.

In summary, in this study, we established an STZ-induced diabetes model and found that teneligliptin significantly inhibited cardiac hypertrophy and reduced fractional shortening and the ejection fraction induced by diabetes. Through animal in vivo and cell in vitro experiments, we found that teneligliptin can improve DCM through NLRP3 pathway, which take to us that cell pyroptosis plays an important role in the improvement of DCM by treatment with teneligliptin. Future study should performed by NLPR3 knocking down and overexpressing to verified its direct effect, which is the main limitations of this study.

CONCLUSION

Overall, teneligliptin mitigated DCM by mitigating activation of the NLRP3 inflammasome, which suggests that in future clinical work, teneligliptin may treat diseases involving the NLRP3 pathway.

ARTICLE HIGHLIGHTS

Research background

Diabetic cardiomyopathy (DCM), which is a complication of diabetes, poses a great threat to public health. Recent studies have confirmed the role of NLRP3 (NOD-like receptor protein 3) activation in DCM development through the inflammatory response. Teneligliptin is an oral hypoglycemic dipeptidyl peptidase-IV inhibitor used to treat diabetes. Teneligliptin has recently been reported to have anti-inflammatory and protective effects on myocardial cells.

Research motivation

This study examined the therapeutic effects of teneligliptin on DCM in diabetic mice.

Research objectives

Teneligliptin improves DCM by means of NLRP3, providing a new target for teneligliptin in the treatment of DCM.

Research methods

Streptozotocin (STZ) was administered to induce diabetes in mice, followed by treatment with 30 mg/kg teneligliptin. Small animal ultrasonic apparatus was used to measure mice heart function, The levels of creatine kinase-MB (CK-MB), aspartate transaminase (AST), lactate dehydrogenase (LDH), and interleukin (IL)-1β were detected by ELISA, cardiomyocytes areas were measured by HE staining, Real-time PCR was used to measure NOX-4 mRNA expression, western blot was used to measure NOX-4, NLRP3, caspase-1, AMPK, p-AMPK protein expression level, primary cardiomyocytes was isolated in neonatal mice. The data are presented as the mean ± SD and were analyzed using one-way analysis of variance with GraphPad Prism software 6.0. $P < 0.05$ was considered to indicate a statistically significant difference.

Research results

Marked increases in cardiomyocyte area and heart weight/tibia length; reductions in fractional shortening, ejection fraction, and heart rate; increases in CK-MB, AST, and LDH levels; and upregulated NADPH oxidase 4 were observed in diabetic mice, all of which were significantly reversed by teneligliptin. Moreover, NLRP3 inflammasome activation and increased release of IL-1β in diabetic mice were inhibited by teneligliptin. Primary mouse cardiomyocytes were treated with high glucose (30 mmol/L) with or without teneligliptin (2.5 or 5 μM) for 24 h. NLRP3 inflammasome activation and the increases in CK-MB, AST, and LDH levels in glucose-stimulated cardiomyocytes were markedly inhibited by teneligliptin, and AMP (p-adenosine 5′-monophosphate)-p-AMPK (activated protein kinase) levels were increased. Furthermore, the beneficial effects of teneligliptin on hyperglycaemia-induced cardiomyocytes were abolished by the AMPK signaling inhibitor compound C.

Research conclusions

Overall, teneligliptin mitigated DCM by mitigating activation of the NLRP3 inflammasome.

Research perspectives

Our study suggests that in future clinical work, teneligliptin may treat diseases involving the NLRP3 pathway.

FOOTNOTES

Co-corresponding authors: Zhen-Huan Chen and Heng-Li Lai.

Author contributions: Zhang GL, Chen ZH, Lai HL designed the research study; Liu Y, Liu YF, Huang XT performed the research; Zhang GL, Chen ZH, Tao Y, and Lai HL analyzed the data and wrote the manuscript; all authors have read and approve the final manuscript. Lai HL and Chen ZH contributed equally to this work as co-corresponding authors. They made equally important contributions in the process of design, submission and revision, and interpreted all the research data.

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Institutional animal care and use committee statement: The study was reviewed and approved by the Jiangxi Provincial People's Hospital Institutional Review Board (Approval No. KT089).

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ORIGINAL ARTICLE

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Novel insights into immune-related genes associated with type 2 diabetes mellitus-related cognitive impairment

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Abstract

BACKGROUND

The cognitive impairment in type 2 diabetes mellitus (T2DM) is a multifaceted and advancing state that requires further exploration to fully comprehend. Neuroinflammation is considered to be one of the main mechanisms and the immune system has played a vital role in the progression of the disease.

AIM

To identify and validate the immune-related genes in the hippocampus associated with T2DM-related cognitive impairment.

METHODS

To identify differentially expressed genes (DEGs) between T2DM and controls, we used data from the Gene Expression Omnibus database GSE125387. To identify T2DM module genes, we used Weighted Gene Co-Expression Network Analysis. All the genes were subject to Gene Set Enrichment Analysis. Protein-protein interaction network construction and machine learning were utilized to identify three hub genes. Immune cell infiltration analysis was performed. The three hub genes were validated in GSE152539 *via* receiver operating characteristic curve analysis. Validation experiments including reverse transcription quantitative real-

time PCR, Western blotting and immunohistochemistry were conducted both *in vivo* and *in vitro*. To identify potential drugs associated with hub genes, we used the Comparative Toxicogenomics Database (CTD).

RESULTS

A total of 576 DEGs were identified using GSE125387. By taking the intersection of DEGs, T2DM module genes, and immune-related genes, a total of 59 genes associated with the immune system were identified. Afterward, machine learning was utilized to identify three hub genes (*H2-T24*, *Rac3*, and *Tfrc*). The hub genes were associated with a variety of immune cells. The three hub genes were validated in GSE152539. Validation experiments were conducted at the mRNA and protein levels both *in vivo* and *in vitro*, consistent with the bioinformatics analysis. Additionally, 11 potential drugs associated with *RAC3* and *TFRC* were identified based on the CTD.

CONCLUSION

Immune-related genes that differ in expression in the hippocampus are closely linked to microglia. We validated the expression of three hub genes both *in vivo* and *in vitro*, consistent with our bioinformatics results. We discovered 11 compounds associated with *RAC3* and *TFRC*. These findings suggest that they are co-regulatory molecules of immunometabolism in diabetic cognitive impairment.

Key Words: Bioinformatics analysis; Type 2 diabetes mellitus; Cognitive impairment; Hippocampus; Immune; Microglia

Core Tip: Using GSE125387, we identified differentially expressed genes in the hippocampus of T2DM mice and controls. Fifty-nine genes were identified through functional enrichment analysis and protein-protein interactions analysis. Machine learning was utilized to identify three hub genes (*H2-T24*, *Rac3*, and *Tfrc*). And the three hub genes were validated in GSE152539. Validation experiments were conducted at the mRNA and protein levels both *in vivo* and *in vitro*. Additionally, 11 potential compands associated with *RAC3* and *TFRC* were identified based on the Comparative Toxicogenomics Database. The findings provide new insights into the treatment of T2DM-related cognitive impairment.

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INTRODUCTION

The prevalence of type 2 diabetes mellitus (T2DM) is rapidly increasing due to lifestyle changes and the aging population. Over the past few years, an increasing number of researches have emphasized the connection between T2DM and cognitive impairment[1]. The severity of cognitive impairment in T2DM may range from mild cognitive decline to more severe forms, including dementia and Alzheimer's disease (AD)[2]. Cognitive impairment in T2DM is a progressively advancing condition. Once upon diagnosis, there is no effective treatment currently available. The pathophysiology of the condition is multifactorial and there are still areas yet to be fully investigated.

Immune system abnormalities play a pivotal role in the pathogenesis of T2DM. Dysregulation of inflammation and immune responses is intricately linked to insulin resistance and beta cell dysfunction[3]. Furthermore, alterations in the expression of immune-related genes may also serve as crucial determinants in the development of diabetes-associated cognitive impairment. Therefore, a complete understanding of the involvement of these immune genes in the cognitive impairment of individuals with T2DM is imperative.

Bioinformatics is used to screen differences at multiple levels from microarray or high-throughput sequencing data, between patients and healthy individuals. Compared to the traditional experimental methods, bioinformatics can explore the hidden molecular mechanisms of diseases, and is regarded as a highly effective research method. A study has discovered a shared biological connection between T2DM and AD through bioinformatics analysis. This connection is strongly associated with synaptic vesicle function and the MAPK signaling pathway[4]. Additionally, the immune system has been found to play a significant role in this link[5]. In a recent investigation, it was discovered that both AD and Metabolic syndrome exhibit the presence of immune cell infiltrations[6]. Furthermore, the shared genes implicated in numerous metabolic pathways are closely linked to diverse immune cells. However, there are few studies on diabetic cognitive impairment using the hippocampus for bioinformatics analysis. There are no bioinformatics studies about immune-related genes and diabetic genes analyzed in cognitive impairment. Bioinformatics approaches may provide us with novel molecules associated with diabetic cognitive impairment that have not been fully studied.

Our study employed advanced bioinformatic methods to conduct a comprehensive analysis of immune-related genes, aiming to elucidate the regulatory mechanisms of these genes on cognitive function in individuals with T2DM and explore their underlying pathogenic pathways. Through bioinformatics, we identified differentially expressed genes

(DEGs) in the hippocampus of diabetic and normal control mice. Meanwhile, we identified module genes most strongly associated with T2DM. We examined the role of immune-related genes in the progression of cognitive impairment linked to T2DM by conducting functional enrichment analysis, protein-protein interaction (PPI) analysis, and analysis of immune cell infiltration. Afterward, machine learning was utilized to identify three hub genes. We also identified the potential drugs associated with hub genes. In the end, we confirmed the expression of these three genes in mice and BV2 cells. Through these insights, we aspire to provide valuable information for the development of more efficacious treatment strategies and the enhancement of the quality of life of patients with T2DM-related cognitive impairment.

MATERIALS AND METHODS

Data collection and processing

The Gene Expression Omnibus (GEO) database[7] provided the datasets GSE125387 and GSE152539, which are associated with T2DM. The dataset GSE125387 consists of high-throughput sequencing data obtained from the hippocampus tissues of db/db mice (a mouse model for T2DM; n = 10) and db/m mice (control mice; n = 11). The Morris Water Maze test has validated distinct cognitive abilities in the two groups of mice, indicating that db/db mice exhibit deficiencies in learning and memory[8]. GSE152539 consists of microarray expression data from hippocampus tissues of mice with high-fat diet-fed (HFD) for 12 months (diabetic model mice; n = 3) and mice with normal control diet-fed (n = 3)[9]. GSE125387 served as the primary dataset for analysis, while GSE152539 was utilized for hub gene validation.

Genes associated with immunity were acquired from Immunology Database and Analysis Porta (ImmPort)[10] and Mouse Genome Informatics (MGI)[11]. ImmPort can be accessed at https://www.immport.org. and MGI at http://www.infor-matics.jax.org.

Analysis of differential expression genes

With GSE125387, we performed DEGs analysis by converting FPKM data into TPM data. The analysis for Principal Component Analysis (PCA) was conducted using the R software package called "stats"[12]. To identify DEGs between the experimental group (db/db) and control group (db/m), we utilized the R package "limma" for differential analysis[13]. In particular, we used $|\text{FoldChange}| > 1.2$ and FDR < 0.05 as filtering criteria. A volcano plot was generated using the R package "ggplot2"[14], and a heat map plot was created using the "ComplexHeatmap" package[15].

Functional enrichment analysis

To conduct the functional enrichment analysis on DEGs, we utilized Metascape (metascape. org/)[16]. The analysis covered different platforms like Reactome[17], Gene Ontology (GO)[18,19], Kyoto Encyclopedia of Genes and Genomes (KEGG)[20], and Wiki pathways[21]. The focus of the analysis was on DEGs, module genes, and immune-related DEGs.

The examination of potential biological functions and pathways in the hippocampus for both T2DM and normal groups within the predefined gene set was conducted using Gene Set Enrichment Analysis (GSEA)[22]. All the genes were subject to analysis. The gene sets identified as "M2 curated gene sets" were obtained from the MSigDB database at https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp. GSEA was implemented using the "clusterProfiler" package[23].

Weighted gene co-expression network analysis

Initially, we computed the deviation of every gene utilizing the gene expression patterns from GSE125387 and eliminated the lowest 75% of genes. To eliminate outliers in genes and samples, the goodSamplesGenes method from the R package "WGCNA" was employed[24]. Afterward, we built a co-expression network with a scale-free property. The co-expression network was built using various criteria, which involved employing a soft thresholding function set at a power of 3. This function followed the scale-free topology criterion and yielded an independent index with an R^2 value of 0.85. Additionally, a minimum of 50 genes were required for each module in conjunction with the dynamic tree-cut method used for module merging, and a threshold of 0.5 was established. We set the sensitivity to 2, ultimately yielding 14 co-expression modules. Further analysis involved the use of Pearson correlation to examine any potential correlation between modules and groups. Moreover, an analysis of functional enrichment was conducted, as previously described.

Identification of immune-related DEGs

To identify immune-related DEGs, we cross-analyzed DEGs, key module genes found through Weighted Gene Co-Expression Network Analysis (WGCNA), and immune-related genes. The overlapping genes were visually presented using the R package "VennDiagram"[25]. Moreover, an analysis of functional enrichment was conducted, as previously described.

Protein-protein interaction network construction

In order to examine the connections among protein-coding genes, we used the STRING database (string-db.org/)[26], with a specified minimum interaction score of 0.400. Nodes obtained from STRING were subsequently modified using Cytoscape software (3.9.1), and key interacting genes were identified with the aid of the CytoHubba plugin. The top 15 genes were independently ranked by Maximal Clique Centrality (MCC), Density of Maximum Neighborhood Component (DMNC) and Maximum Neighborhood Component (MNC).

Machine learning

For diagnosis, two additional machine learning algorithms were employed to further screen candidate genes. The technique of Lasso regression was utilized for variable selection and regularization, thereby enhancing predictive accuracy[27]. For this research, we employed the R software package "glmnet" to perform regression analysis with the Lasso technique[28]. In addition, we established a 3-fold cross-validation in order to acquire the most suitable model. The value of Lambda was adjusted to 0.05. In the meantime, the Random Forest (RF) algorithm was utilized due to its lack of limitations on variable conditions and ability to offer improved accuracy, sensitivity, and specificity[29]. The RF analyses were conducted through the R package "randomForest"[30]. Further diagnosis involved considering the hub genes obtained from the combination of Lasso and RF cross genes.

Receiver operating characteristic evaluation

Receiver operating characteristic (ROC) curve analysis was utilized to evaluate the diagnostic and discriminative significance of immune-related genes in cognitive impairment associated with T2DM. In order to measure the diagnostic worth, we computed the area under the curve (AUC) and assessed its significance by determining the 95% confidence interval (95%CI). The data was analyzed using the R package "pROC" to conduct ROC analysis[31]. The GSE152539 dataset was employed as the external validation dataset.

Immune cell infiltration analysis

The analysis of tissue gene expression profiles using CIBERSORT, a computational technique, allows for the determination of the quantity of various immune cells[32]. The immune infiltration of GSE125387 data was computed in this investigation by utilizing the markers of 25 immune cells in mice[33]. For the analysis of immune cell infiltration, we utilized the dataset of mouse immune genes as the gene feature[33] through the CIBERSORTx website (https://cibersortx.stanford.edu/index.php/). We provided Supplementary Table 1 with the markers of 25 immune cells as the signature matrix. Bars were used to represent the distribution of immune cells in various samples. Comparisons between the proportions of various immune cell types in the diabetic and control groups were made using violin plots. To illustrate the connection between the hub genes and immune cells, we conducted a pairwise correlation analysis using the "Spearman" method. The outcomes of the analysis were then presented through heat maps utilizing the R package "ggplot2"[14].

Potential drug analysis

This study aims to analyze potential drugs that are effective in treating cognitive impairment in individuals with T2DM. *Rac3* and *Tfrc* were employed to identify potentially efficacious medications for Comparative Toxicogenomics Database (CTD) (https://ctdbase.org/) correspondingly. Afterward, we utilized PubChem (https://pubchem.ncbi.nlm.nih.gov/) to obtain the molecular formulas and two-dimensional structures of potential medications, aiding in the investigation of drugs.

Experimental animals and ethics

Male BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J homozygous Lepr$^{db/db}$ mice were diabetic, and heterozygous Lepr$^{db/m}$ mice were used as controls (denoted as db/db and db/m in the text) in this study. As a diabetic model, the mice we used were consistent with the dataset GSE125387. A total of 9 male db/db mice aged six weeks and 9 male db/m mice aged six weeks were acquired from Jiangsu Hhuachuang sinoPharmaTechCo., Ltd. Mice were raised in the Animal Research Center of Shandong University and housed in an environment of SPF level standards. The mice were kept in a chamber where the temperature was regulated within the range of 22°C to 25°C. The humidity is regulated within the range of 50% to 60% and the environment follows a 12-h cycle alternating between light and darkness. Autoclaving water was used for drinking and food was taken ad libitum. After being fed a regular diet for 24 wk, the mice were euthanized in order to collect brain tissue. The bilateral hippocampus of 3 mice from each group randomly selected was isolated for total RNA and protein extraction, respectively. While the cerebral hemispheres of remaining mice from each group were made into paraffin sections.

The Experimental Animal Ethics Review Committee of Qilu Medical College of Shandong University granted approval for our animal protocol (No. 23001). The study adhered to principles that support the protection, well-being, and ethical treatment of animals, and it also complied with applicable national regulations concerning the welfare of laboratory animals.

Cell line culture and treatment

The BV-2 cell line (mice microglia) was cultured in DMEM/high-glucose medium (Gibco, United States) supplemented with 10% fetal bovine serum (Gibco, United States), 1% penicillin, and streptomycin. The cells were incubated at 37°C with 5% CO_2. Palmitic acid (PA) is a common saturated fatty acid. It is the main component of HFD and it has been found increased in the circulation of obese and diabetic people. PA has been studied in various biological contexts including inflammation, metabolic disorders, and cell signaling. In the central nervous system, PA has been associated with inflammatory responses. PA is recognized as a T2DM model *in vitro*, such as in BV2 cells[34], β cells[35], and skeletal muscle cells[36]. Changes in the cerebral gene expression profiles seemed to be specific in the T2DM model, as no such alterations were found in the type 1 diabetes mellitus model[37]. So we chose the high-fat model instead of the high-glucose model. BV-2 cells were treated with 0.4 mmol/L PA (Sigma-Aldrich, United States) for a duration of 24 h, followed by extraction of total RNA or protein.

Reverse transcription quantitative real-time PCR

Total RNA was extracted from hippocampus or cells using the Total RNA Isolation Kit (Vazyme RC101) following the provided instructions. Reverse transcription was then performed with a Reverse Transcription Kit (Vazyme R323-01) and Thermal Cycler (Life Technologies, 2720). Q-PCR was done with SYBR Green (Vazyme Q711-02/03) and Quantitative real-time PCR system (Roche, LightCycler480). The primer sequences were created and compared using the Primer-BLAST website (ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome), and subsequently synthesized by Beijing Tsingke Biotech Co., Ltd. The expression of the target gene, relative to the β-Actin gene, was represented as $2^{-\Delta\Delta Ct}$.

Western blotting

The samples were extracted from hippocampus tissue or cells and subsequently boiled in a loading buffer for 10 min. Protein separation was accomplished through SDS-PAGE (Epizyme, PG113). Primary antibodies, such as β-TUBLIN (Dilution 1:10000, Abways AB0039), RAC3 (Dilution 1:2000, Abcam ab129062), and TFRC (Dilution 1:1000, BOSTER PB9233), were incubated with the PVDF membranes overnight at 4°C. Afterward, the membranes were exposed to a secondary antibody (Dilution 1:10000, ZSGB-BIO ZB2305, ZB2301) for a duration of 2 h at room temperature. Subsequently, the electrochemiluminescence system was employed to detect the presence.

Immunohistochemistry

The paraffin sections were dewaxed, rehydrated, and subjected to antigen retrieval. To deactivate the natural peroxidase, a solution of 3% hydrogen peroxide was applied for a duration of 15 minutes. Following a 1-hour treatment with 5% BSA (bovine serum albumin), the slices were incubated overnight with primary antibodies. These antibodies included RAC3 (Dilution 1:100, Abcam ab129062) and TFRC (Dilution 1:100, BOSTER PB9233). On the next day, the paraffin sections were incubated with a secondary antibody (Genetech GK600505) at room temperature for a duration of 2 h. In the end, the slides underwent staining with a DAB Detection Kit (Genetech GK600505) and were subsequently counterstained with hematoxylin.

Data analysis

Three independent experiments were conducted and the data were presented as the mean ± SEM. Confirmation of data normality was established through the utilization of the Shapiro-Wilk test. To assess the distinction between two groups, Student's t-test was employed for data that followed a normal distribution. The Wilcoxon rank sum test was employed for data that did not follow a normal distribution. A statistically significant difference was defined as a P value < 0.05. Data analysis was performed with the use of R software (4.2.3) and Prism 9.

RESULTS

The Process of PCA and detection of DEGs

The flow chart for this study is illustrated in Figure 1. Using GSE125387, we identified DEGs between T2DM and control mice. Meanwhile, we identified module genes most strongly associated with T2DM using WGCNA. All the genes were subject to GSEA for functional enrichment analysis. By taking the intersection of DEGs, T2DM module genes, and immune-related genes, a total of 59 genes associated with the immune system were identified. Afterward, PPI and machine learning (Lasso regression and RF) were utilized to identify three hub genes (*H2-T24*, *Rac3*, and *Tfrc*). Immune cell infiltration analysis was performed. The three hub genes were validated in GSE152539. Validation experiments were conducted at the mRNA and protein levels both *in vivo* and *in vitro*. Additionally, 11 potential drugs associated with *RAC3* and *TFRC* were identified based on CTD.

The PCA indicated that the db/db group and db/m group were distinctly separated into two separate groups (Figure 2A). A total of 576 DEGs were detected, consisting of 214 genes showing upregulation and 362 genes exhibiting downregulation (Figure 2B). A heatmap displayed the most significant DEGs (Figure 2C).

ImmPort and MGI provided a collection of 4142 genes related to mouse immune system.

Analysis of functional enrichment

Functional enrichment analysis was conducted using DEGs between db/db group and db/m group. The results demonstrated that DEGs were enriched in the "Microglia pathogen phagocytosis pathway" (WikiPathways); "positive regulation of endocytosis", "circulatory system process", "positive regulation of immune response", "negative regulation of cell population proliferation", "immune effector process" and "behavioral response to ethanol"(GO); "platelet activation, signaling and aggregation" and "metabolism of amine-derived hormones" (Reactome); "VEGF signaling pathway - Mus musculus (house mouse) " (KEGG; Figure 2D). GSEA results revealed that, in comparison to the db/m control group, "overlap between signal transduction pathways contributing to LMNA laminopathies" and "iron uptake and transport" pathways were upregulated in the db/db group (Figure 2E); "neuroactive ligand-receptor interaction" and "collagens" were downregulated (Figure 2F). The detailed enrichment items are listed in Supplementary Table 2 and 3.

Weighted gene co-expression network analysis

To approximate the scale-free structure of the network, we utilized a soft thresholding value of 3 (Figure 3A and B). We combined the top 25% of genes with the greatest variability into 14 co-expression modules by means of clustering

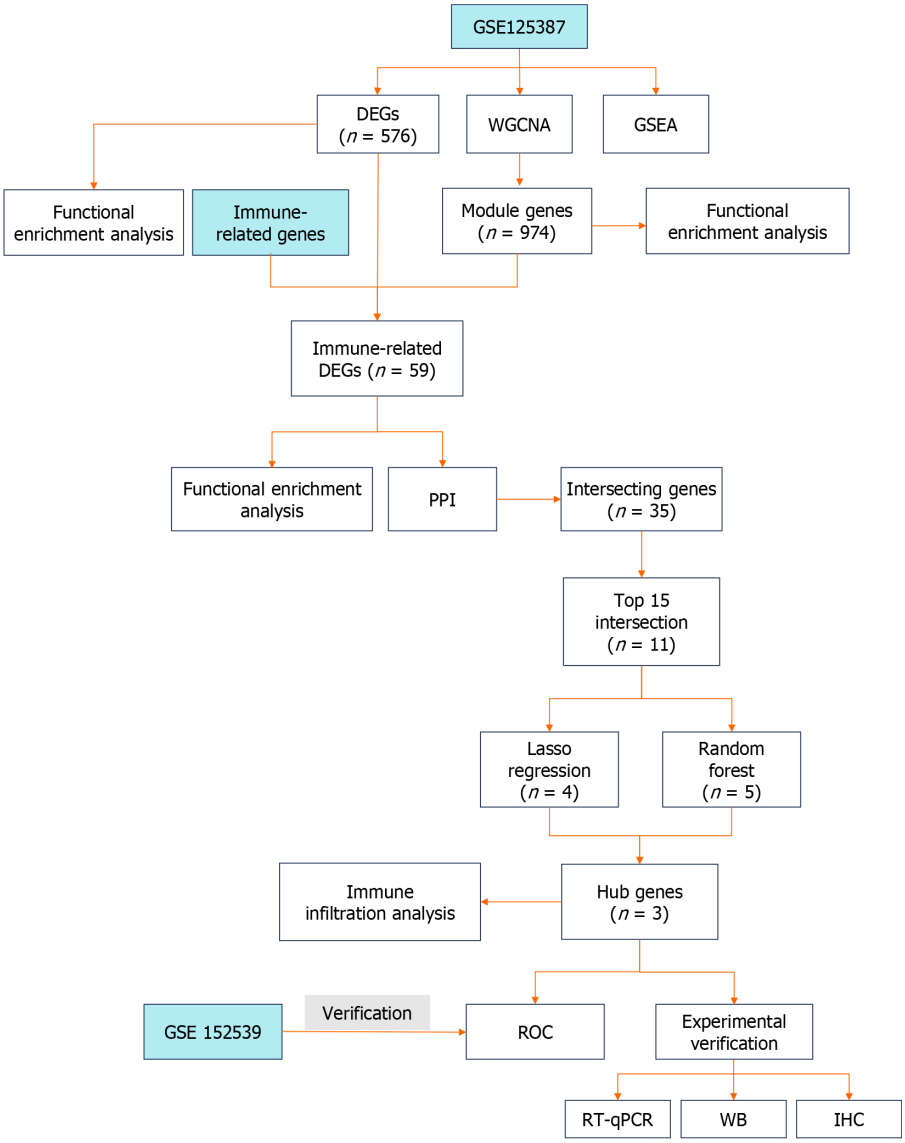
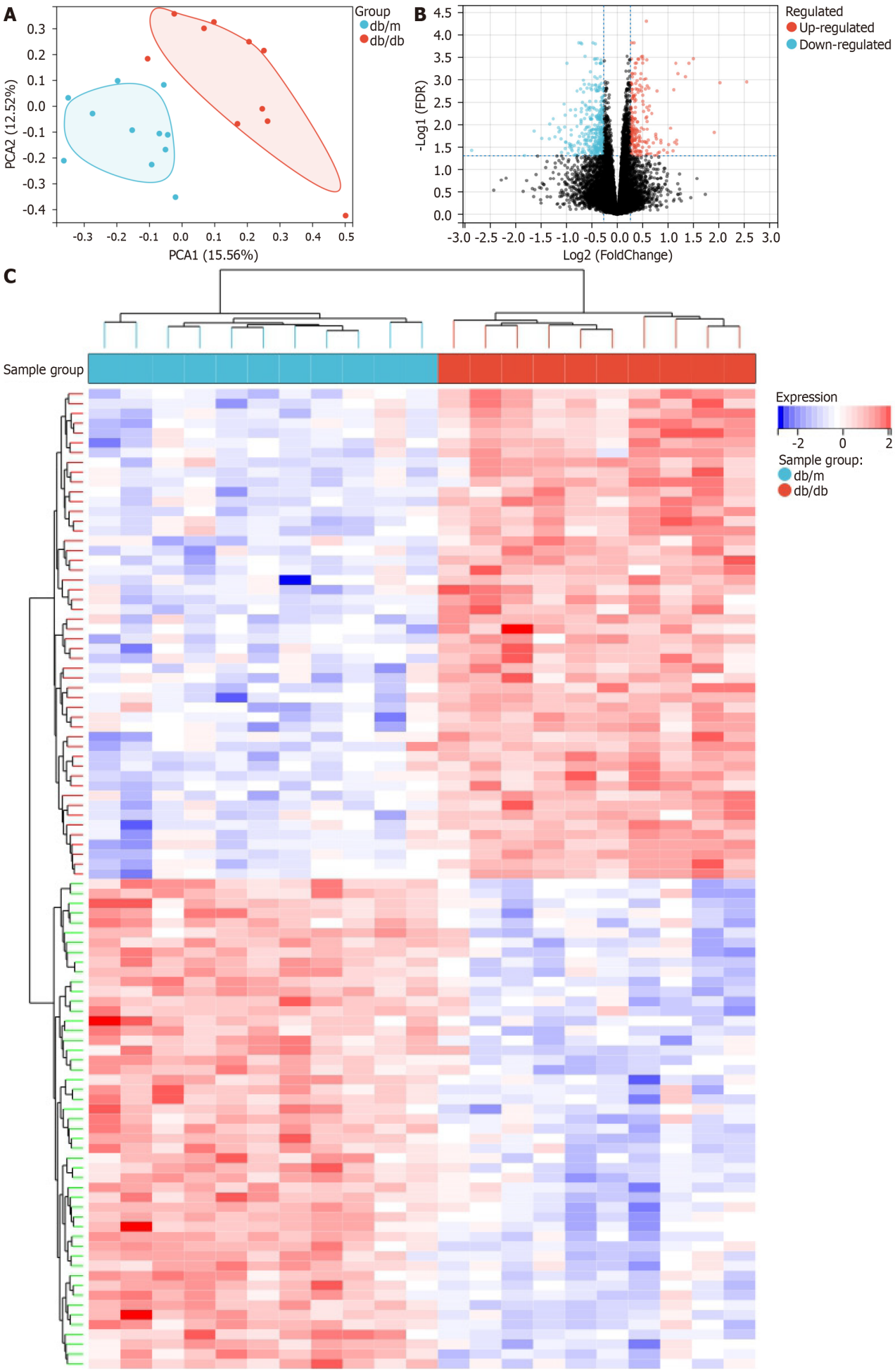


Figure 1 Flow chart of the methods used in this study. DEGs: Differentially expressed genes; WGCNA: Weighted Gene Co-Expression Network Analysis; GSEA: Gene Set Enrichment Analysis; PPI: Protein-protein interaction; ROC: Receiver operating characteristic; RT-qPCR: Reverse transcription quantitative real-time PCR; WB: Western blotting; IHC: Immunohistochemistry.

(Figure 3C). Next, we conducted a Pearson correlation analysis to explore the connections between genes that define the modules and the traits of the groups. Our findings revealed that the Brown Module, comprising 974 genes, exhibited a significant association with the "group" trait (db/db and db/m) and displayed the strongest correlation (Figure 3D and E). In addition, we carried out functional enrichment analysis for the genes within the Brown Module. It showed the genes were enriched in "Collagen chain trimerization" and "Neuronal System" (Reactome); "behavior", "regulation of membrane potential", "synaptic signaling" and "locomotory behavior" (GO); "Serotonin and anxiety-related events" (WikiPathways; Figure 3F, Supplementary Table 4).

Identification of Immune-related DEGs

The cognitive impairment of T2DM mice (db/db) that we utilized has been confirmed[8]. The above analysis of enrichment indicated a strong association between DEGs and the immune system. In order to explore the connection between cognitive impairment related to diabetes and immunity, we utilized a Venn diagram (Figure 4A) to identify 59 genes that overlapped between DEGs, genes in the Brown Module, and immune-related genes. Figure 4B displays the heat map of the 59 genes. The analysis of gene function enrichment revealed that the immune-related DEGs were highly concentrated in pathways such as "Microglia pathogen phagocytosis pathway" (WikiPathways), "positive regulation of immune response", "regulation of leukocyte cell-cell adhesion", "regulation of neuron death", and "regulation of behavior". Additionally, the pathways "Axon guidance - Mus musculus (house mouse) " and "B cell receptor signaling pathway - Mus musculus (house mouse)" (KEGG) as well as "ER-Phagosome - Mus musculus (house mouse)" (Reactome) were also enriched (Figure 4C and Supplementary Table 5). Summary of enrichment analysis in PaGenBase about the prediction of specific cell types for the 59 immune-related DEGs showed they were predicted in microglia (Figure 4D).


A
Group
db/m
db/db
PCA2 (12.52%)
PCA1 (15.56%)
B
Regulated
Up-regulated
Down-regulated
-Log1 (FDR)
Log2 (FoldChange)
C
Sample group
Expression
Sample group:
db/m
db/db

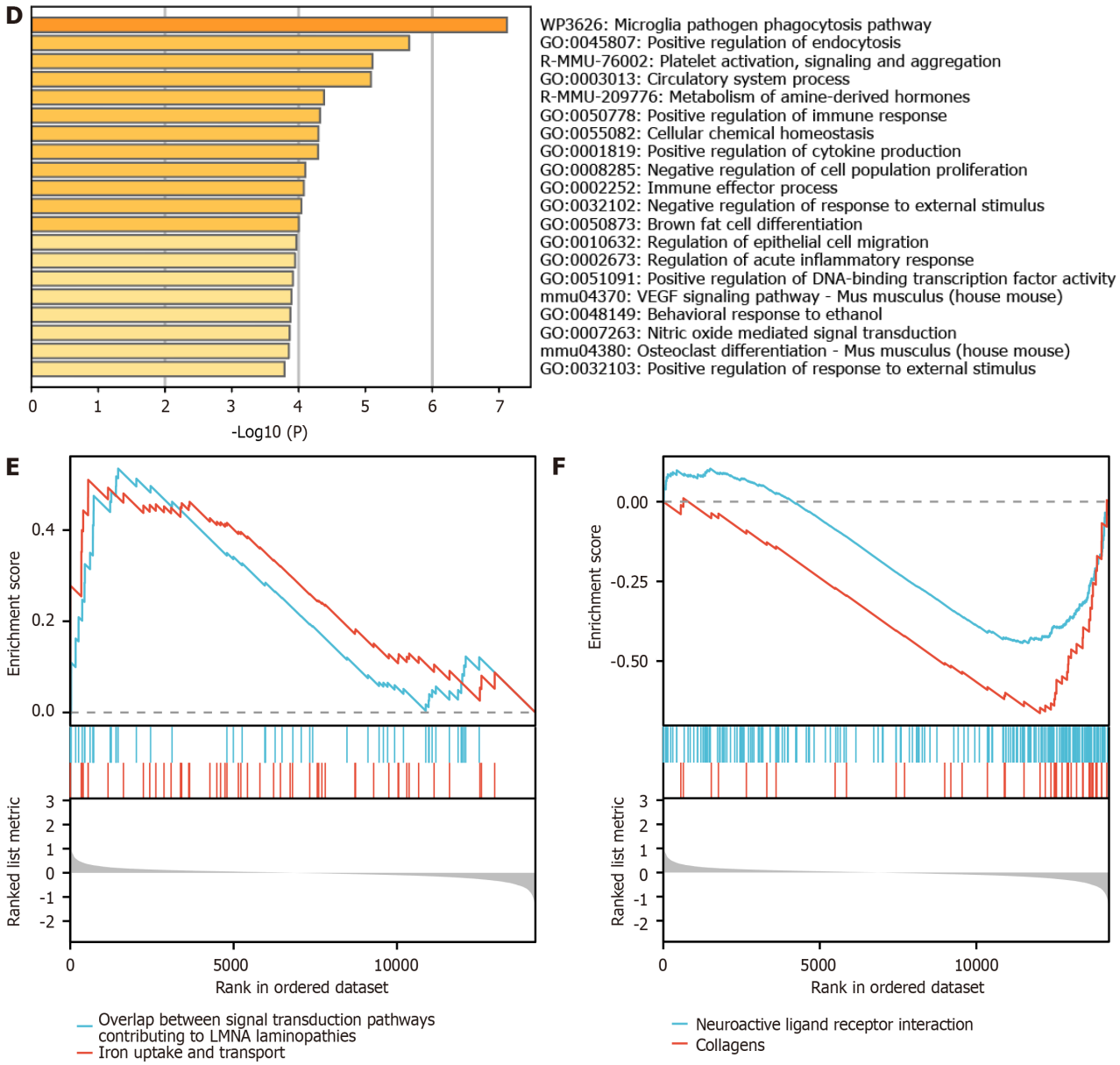


Figure 2 Differentially expressed genes in GSE125387 and functional enrichment analysis. A: Principal component analysis shows that the db/db group and db/m group were distinctly separated; B: The volcano plot illustrates the distributions of differentially expressed genes (DEGs), with 214 genes showing upregulation (represented by red dots) and 362 genes showing downregulation (represented by blue dots). No significantly changed genes are marked as black dots; C: Heat map plot of the most significant DEGs; D: Analysis of functional enrichment for DEGs; E: Gene Set Enrichment Analysis (GSEA) between all the genes showing the up-regulated pathways; F: GSEA between all the genes showing the down-regulated pathways. PCA: Principal component analysis.

PPI network

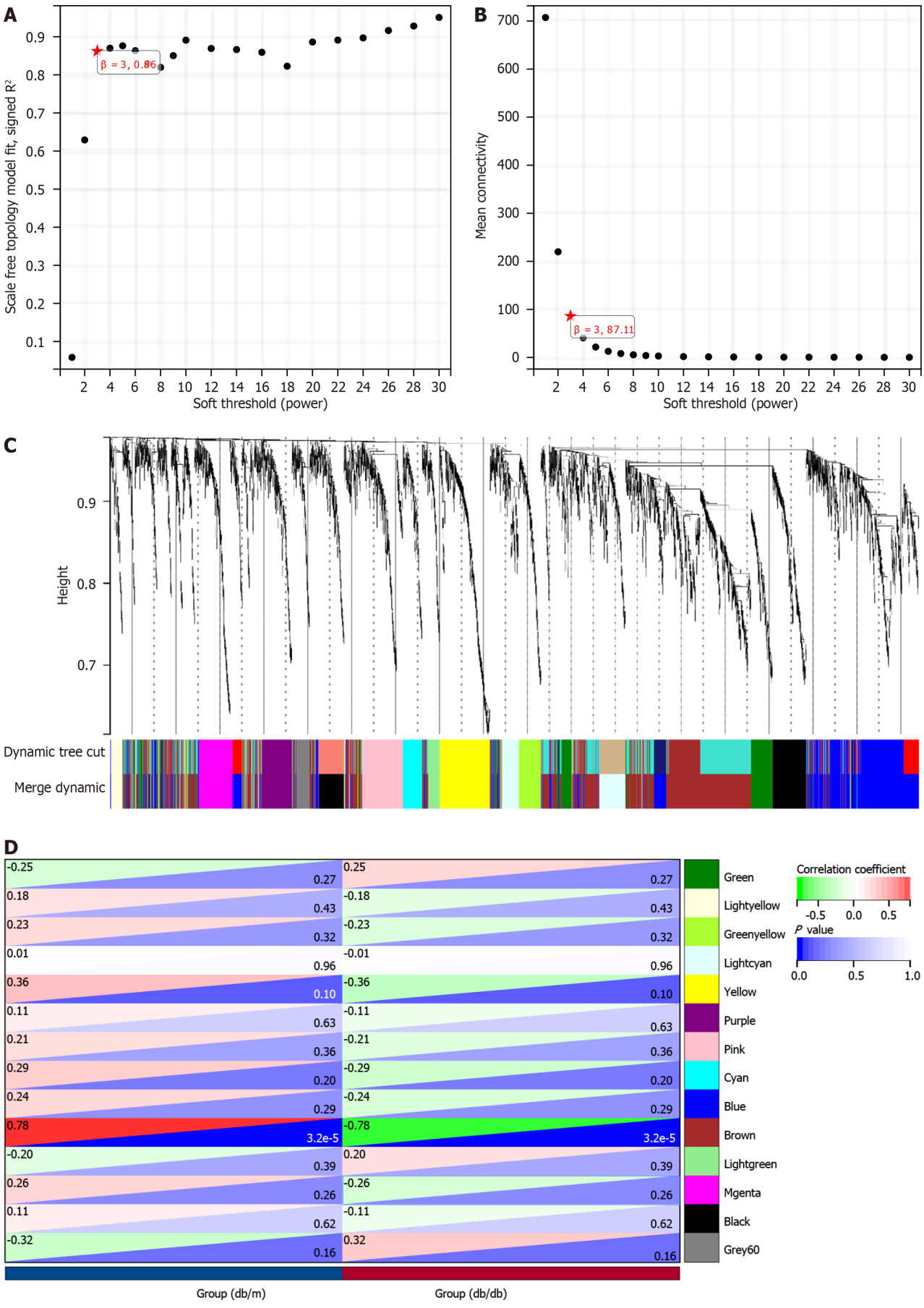
The PPI network is shown in Figure 5A, with 35 genes displaying interaction capabilities. The visualization was performed utilizing the CytoHubba plugin within Cytoscape (Figure 5B). The MCC, DMNC, and MNC methods independently ranked the top 15 genes. The significance of the interaction network increases as the color becomes darker (Figure 5C-E). The overlap of the top 15 genes acquired through the three approaches resulted in 11 genes (Figure 5F).

Identification of hub genes using machine learning

To evaluate the diagnostic significance of potential genes, we utilized Lasso regression and RF machine learning techniques. Figure 6A and B revealed that Lasso regression detected 4 possible biomarker contenders namely *C1qa*, *H2-T24*, *Rac3*, and *Tfrc*. The genes were ranked according to their importance by the RF algorithm (Figure 6C and D). To depict the overlap between the 4 possible contenders in Lasso and the leading 5 genes in RF, a Venn diagram was employed, leading to the identification of 3 genes (*H2-T24*, *Rac3*, and *Tfrc*) for the ultimate validation phase (Figure 6E).

ROC evaluation

In GSE125387, the findings were as follows: *H2-T24* (AUC 1.000, 95%CI: 1.000–1.000), *Rac3* (AUC 0.982, 95%CI: 0.939–1.000), and *Tfrc* (AUC 0.991, 95%CI: 0.966–1.000, Figure 7A-C). Figure 7D displayed the expression levels of these


A
Scale free topology model fit, signed R²
β = 3, 0.86
Soft threshold (power)
B
Mean connectivity
β = 3, 87.11
Soft threshold (power)
C
Height
Dynamic tree cut
Merge dynamic
D
Green
Lightyellow
Greenyellow
Lightcyan
Yellow
Purple
Pink
Cyan
Blue
Brown
Lightgreen
Mgenta
Black
Grey60
Correlation coefficient
P value
Group (db/m)
Group (db/db)

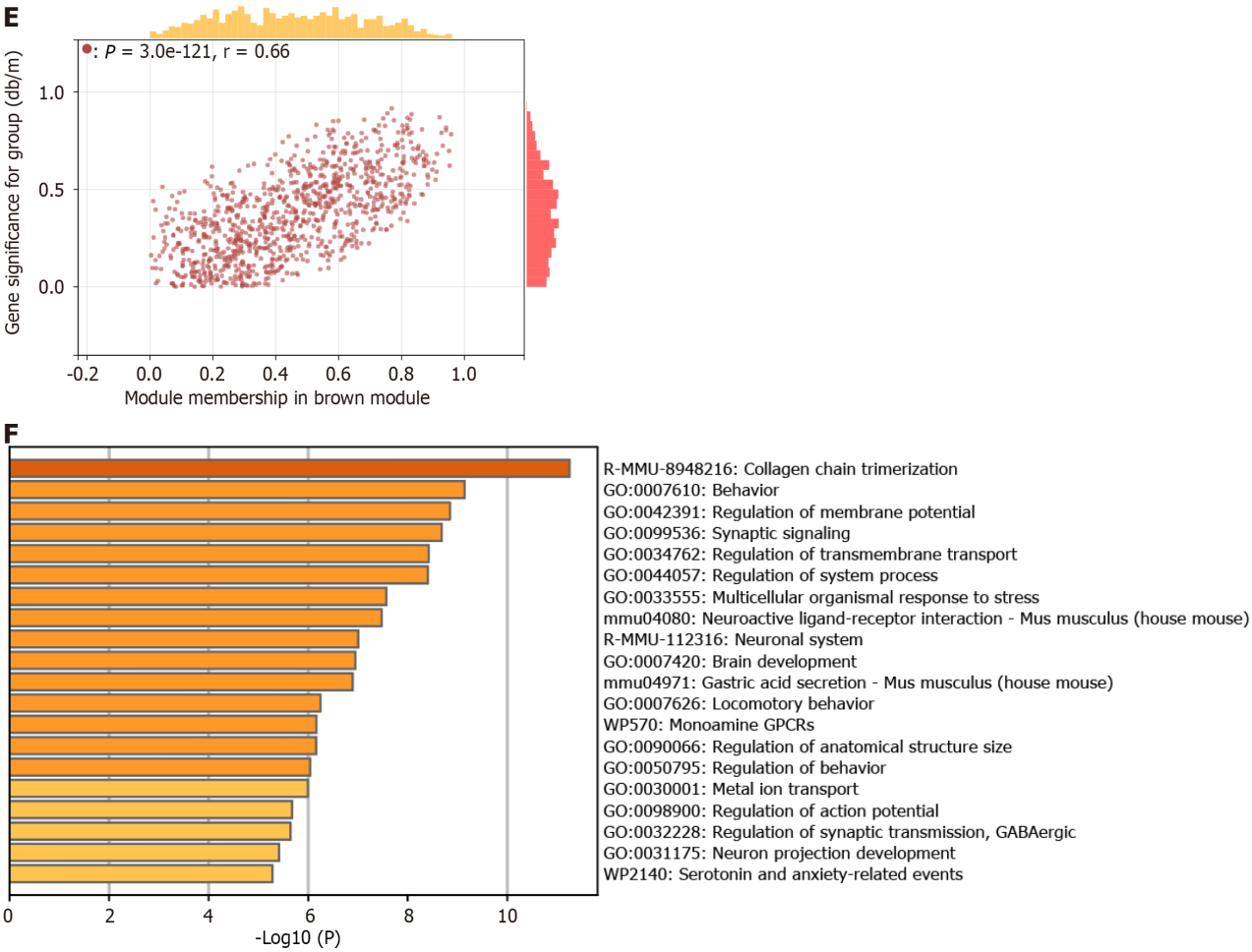


Figure 3 Identification of module genes *via* Weighted Gene Co-Expression Network Analysis. A and B: The soft threshold β = 3 is chosen as the result of the combined analysis of scale independence and average connectivity; C: Different colors represent gene co-expression modules in the gene tree; D: Heatmap illustrating the correlation between modules and type 2 diabetes mellitus (T2DM). There is a significant correlation between the brown module and T2DM; E: The brown module contains 974 genes, and the scatter diagram shows the correlation between membership in the brown module and gene significance for the group; F: Analysis of gene enrichment in the brown module for functional purposes.

three genes in GSE125387. The GSE152539 dataset was employed as the external validation dataset. The findings were as follows: *H2-T24* (AUC 0.667, 95% CI 0.013–1.000), *Rac3* (AUC 1.000, 95%CI: 1.000–1.000), and *Tfrc* (AUC 1.000, 95%CI: 1.000–1.000, Figure 7E-G). In GSE152539, the expression levels of three genes were also confirmed (Figure 7H).

Immune cell infiltration analysis

To gain a deeper understanding of the immune regulation involved in the hippocampus of T2DM, we performed an analysis of the infiltration of immune cells. We utilized 25 mouse immune cells as feature genes to determine the relative abundance of each immune cell in the samples from the db/db and db/m groups. The results were visualized in a bar graph (Figure 8A). According to the violin plot, the level of "M2 Macrophage" (P = 0.082) was higher in the db/db group compared to the db/m group. Conversely, the levels of "T Cells CD4 Follicular" (P = 0.051) and "M0 Macrophage" (P = 0.089) were lower in the db/db group. There is a tendency of effect but it is not statistical. The db/db and db/m groups did not show expression of "T Cells CD8 Actived", "Treg Cells", and "T Cells CD4 Memory" (Figure 8B). Moreover, the correlation heatmap exhibited associations among various immune cell types and 3 hub genes. We observed the infiltration of various immune cells in diabetic mice. The strongest synergistic effect was observed between the categories of "T Cells CD4 Follicular" and "M0 Macrophage" (r = 0.841), with the subsequent highest correlation found between "B Cells Naive" and "M0 Macrophage" (r = 0.709). On the other hand, the most significant competitive impact was observed between "NK Resting" and "NK Actived" (r = -0.796), with "B Cells Naive" and "B Cells Memory" (r = -0.795). A variety of immune cells were linked to *H2-T24*, *Rac3*, and *Tfrc*. The study's correlation analysis revealed a negative association between *H2-T24* and "Th2 Cells". The presence of *Rac3* showed a positive correlation with "M0 macrophage". The association of *Tfrc* with "Neutrophil Cells", "M0 Macrophage", and "T cells CD4 Follicular" was negative, whereas it was positive with "T cells CD4 Naive"(Figure 8C).

Identifying potential drugs

To illuminate the individualized therapy for cognitive impairment in diabetes, researchers identified small molecule drugs targeting *Rac3* and *Tfrc*. 11 associated drugs were identified (Table 1, Figure 9). These drugs mainly affect the

Table 1 Eleven potential compounds were selected by Comparative Toxicogenomics Database

Target gene	Chemical name	Chemical formula	Interaction
RAC3	11-nor-delta(9)-tetrahydrocannabinol-9-carboxylic acid	$C_{21}H_{28}O_4$	Affects methylation[84]/increases abundance
RAC3	Benzo(a)pyrene	$C_{20}H_{12}$	Increases methylation[85]
RAC3	Bis(4-hydroxyphenyl)sulfone	$C_{12}H_{10}O_4S$	Increases methylation[86]
RAC3	Bisphenol A	$C_{15}H_{16}O_2$	Decreases methylation[87]
RAC3	Cannabinoids	$C_{21}H_{30}O_2$	Affects methylation[84]/increases abundance
RAC3	Cyclosporine	$C_{62}H_{111}N_{11}O_{12}$	Decreases methylation[88]
RAC3	Valproic Acid	$C_8H_{16}O_2$	Increases methylation[89]
TFRC	Choline	$C_5H_{14}NO_+$	Affects cotreatment[90]/decreases methylation
TFRC	Folic Acid	$C_{19}H_{19}N_7O_6$	Affects cotreatment[90]/decreases methylation
TFRC	Methionine	$C_5H_{11}NO_2S$	Affects cotreatment[90]/decreases methylation
TFRC	Vinclozolin	$C_{12}H_9C_{12}NO_3$	Increases methylation[91]

Names, molecular formulas and interaction with target genes are shown.

methylation of genes or promoters. It is worth noting that *H2-T24* is not a homologous gene in humans. Therefore, we decided not to proceed with the drug predictions that were planned.

Validation of the expression of hub genes in vivo and in vitro

Supplementary Table 6 displays the primer sequences. Student's t-test was employed for data. The mRNA expression of 3 hub genes (*H2-T24*, *Rac3*, and *Tfrc*) was validated with reverse transcription quantitative real-time PCR (RT-qPCR). In comparison to db/m mice, *H2-T24* and *Rac3* showed a reduction in expression in db/db mice ($P < 0.01$; $P < 0.05$), whereas *Tfrc* demonstrated an elevation in expression in db/db mice ($P < 0.01$; Figure 10A). PA is a common saturated fatty acid. PA was used as a T2DM model *in vitro*. Similar to the findings in mice experiments, *H2-T24* and *Rac3* showed a reduction in expression in the PA group ($P < 0.05$), whereas *Tfrc* demonstrated an elevation in expression in the PA group ($P < 0.05$; Figure 10B). Subsequently, we conducted additional verification of the protein levels of RAC3 and TFRC through *in vivo* and *in vitro* western blotting. The findings indicated that the protein levels of RAC3 and TFRC were in agreement with the mRNA levels ($P < 0.05$) as demonstrated in Figure 10C-F. In the hippocampus of db/db mice, immunohistochemistry revealed a decrease in RAC3 expression and an increase in TFRC expression, as compared to db/m mice (Figure 10G).

DISCUSSION

The worldwide incidence of T2DM is on the rise, occurring concomitantly with target organ damage and poor prognosis, often linked to the occurrence and development of cognitive impairment. Several mechanisms have been proposed to explain the relationship between T2DM and cognitive impairment, including hyperglycemia, insulin resistance, vascular impairment, oxidative stress, and neuroinflammation[3]. Neuroinflammation is a term used to describe the stimulation of glial cells, specifically microglia and astrocytes, leading to the generation of inflammatory cytokines and chemokines within the central nervous system[38]. A study found that knockout of TLR2 protected against diabetes-induced cognitive impairment[39]. Similarly, suppression of NLRP3 enhances cognitive ability and maintains vascular health following stroke in diabetic animals[40]. Hence, it is imperative to conduct additional research to investigate the causes of cognitive impairment in diabetes and discover possible treatment targets.

In this study, several biological information research methods were used to obtain DEGs related to T2DM in mice from GEO high-throughput sequencing datasets. Pathway enrichment analysis showed that DEGs were linked to microglia pathogen phagocytosis, immune inflammation, and collagen synthesis. Our study focused on analyzing the interaction between immune regulation and the development of cognitive impairment in diabetes to explore relevant targets. The results of this research may enhance comprehension regarding immune impairment, neuroinflammatory processes, and their impact on cognitive impairment in individuals with diabetes.

Currently, immune-related genes associated with cognitive impairment have been identified, but there is a lack of bioinformatics studies on diabetic cognitive impairment. In this study, we utilized the immune databases from MGI and ImmPort to identify immune-related DEGs. To further characterize diabetes phenotype genes, we performed a WGCNA analysis. We conducted a cross-analysis of DEGs, key module genes found through WGCNA, and immune-related genes to identify DEGs associated with the immune system. Our research found that immune-related DEGs were highly concentrated in the immune response and phagocytosis pathways. The summary of the enrichment analysis showed that immune-related DEGs were predicted in microglia.

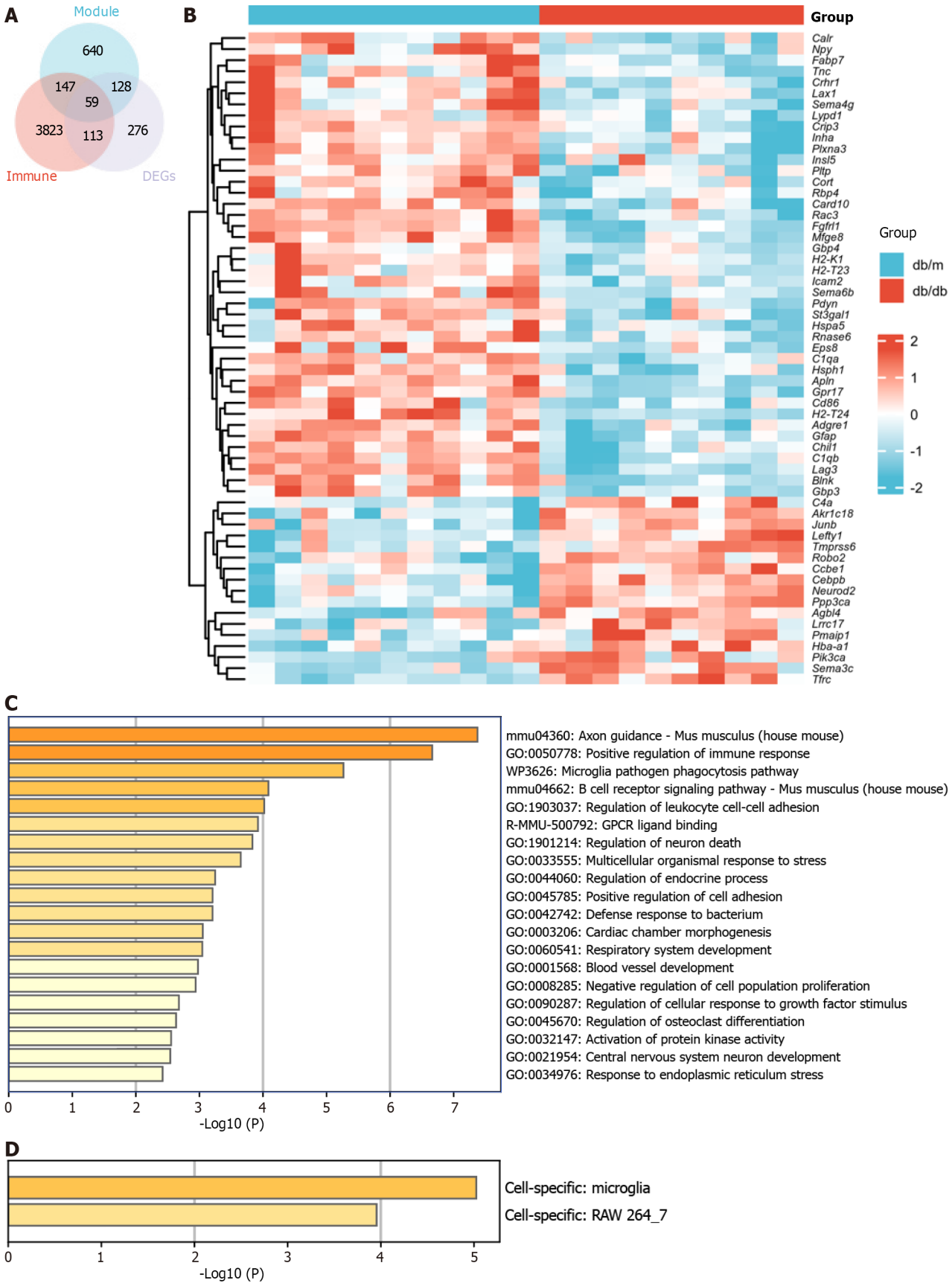


Figure 4 Identification of immune-related differentially expressed genes and functional enrichment analysis. A: The intersection of differentially expressed genes (DEGs), immune-related genes, and brown module genes *via* Weighted Gene Co-Expression Network Analysis reveals a total of 59 identified genes; B: Heat map plot of the 59 immune-related DEGs; C: Functional enrichment analysis was performed on the 59 genes; D: Prediction of specific cell types for the 59 genes. DEGs: Differentially expressed genes.

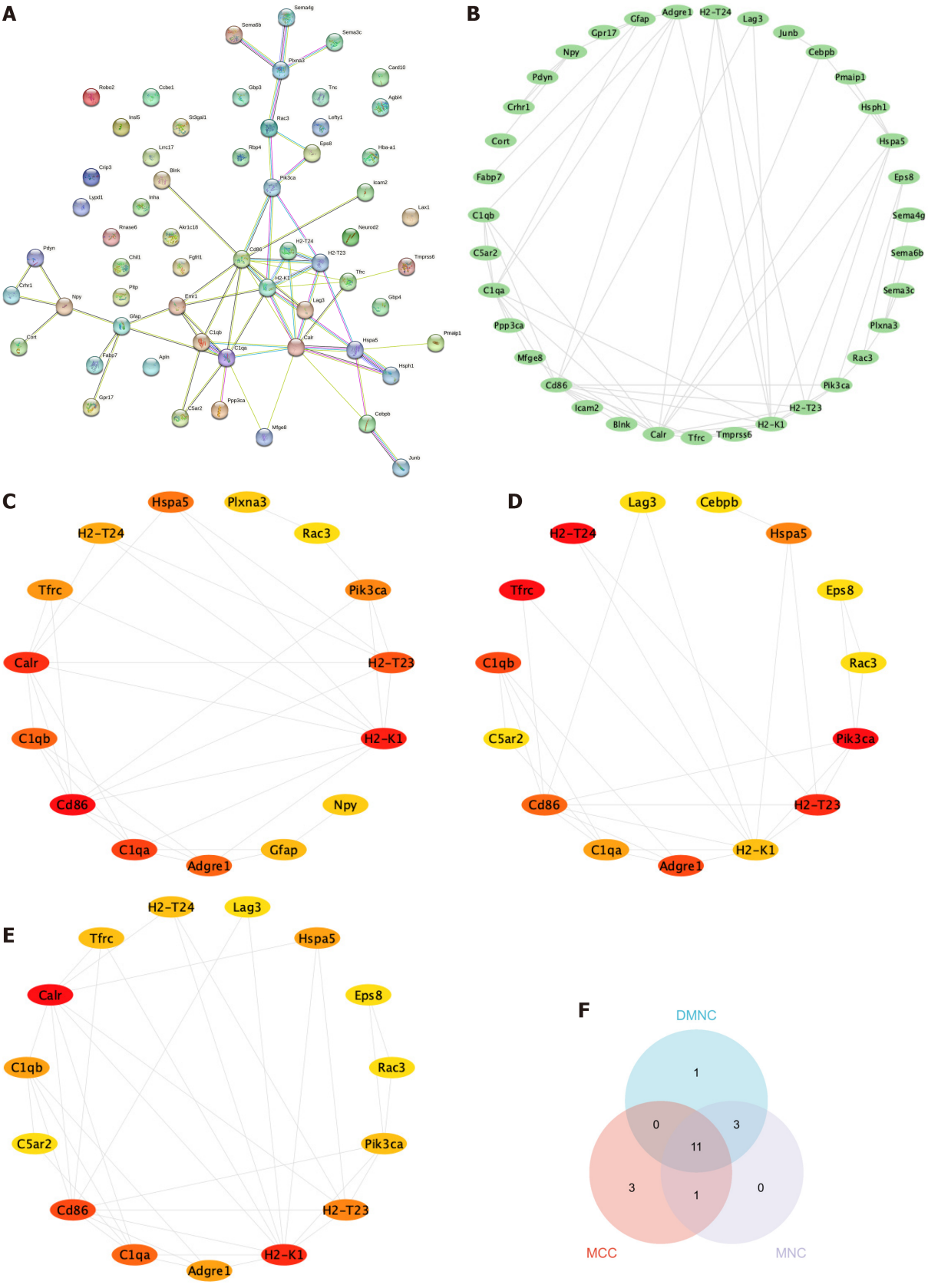


Figure 5 Construction of a network for the interaction between proteins. A and B: The protein-protein interaction network analysis shows that 35 genes have interactions with one another; C-E: Top 15 genes were independently ranked by Maximal Clique Centrality, Density of Maximum Neighborhood Component and Maximum Neighborhood Component methods. The scores were ordered by color from red to yellow; F: The overlap of the top 15 genes obtained from the three methods resulted in a total of 11 genes. MCC: Maximal Clique Centrality; DMNC: Density of Maximum Neighborhood Component; MNC: Maximum Neighborhood Component.

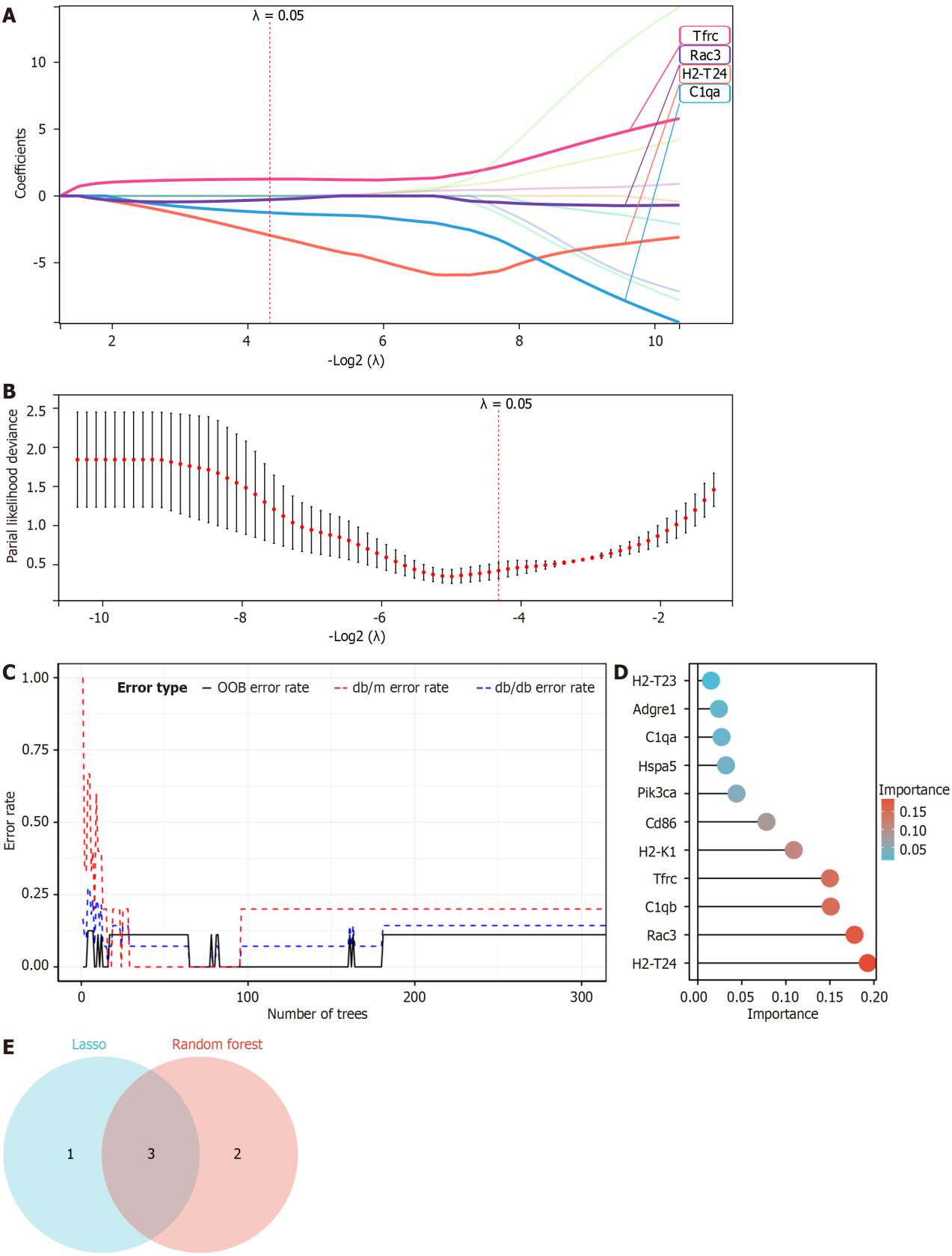


Figure 6 Utilizing machine learning to evaluate potential diagnostic biomarkers in candidate screening. A and B: Screening of biomarkers in the Lasso model. For diagnosis, the most appropriate gene count (n = 4) is the one that corresponds to the lowest point on the curve; C and D: The error is displayed by the random forest algorithm. The importance score is used to rank genes; E: The Venn diagram illustrates that the above two algorithms have identified three potential diagnostic genes: *H2-T24*, *Rac3* and *Tfrc*.

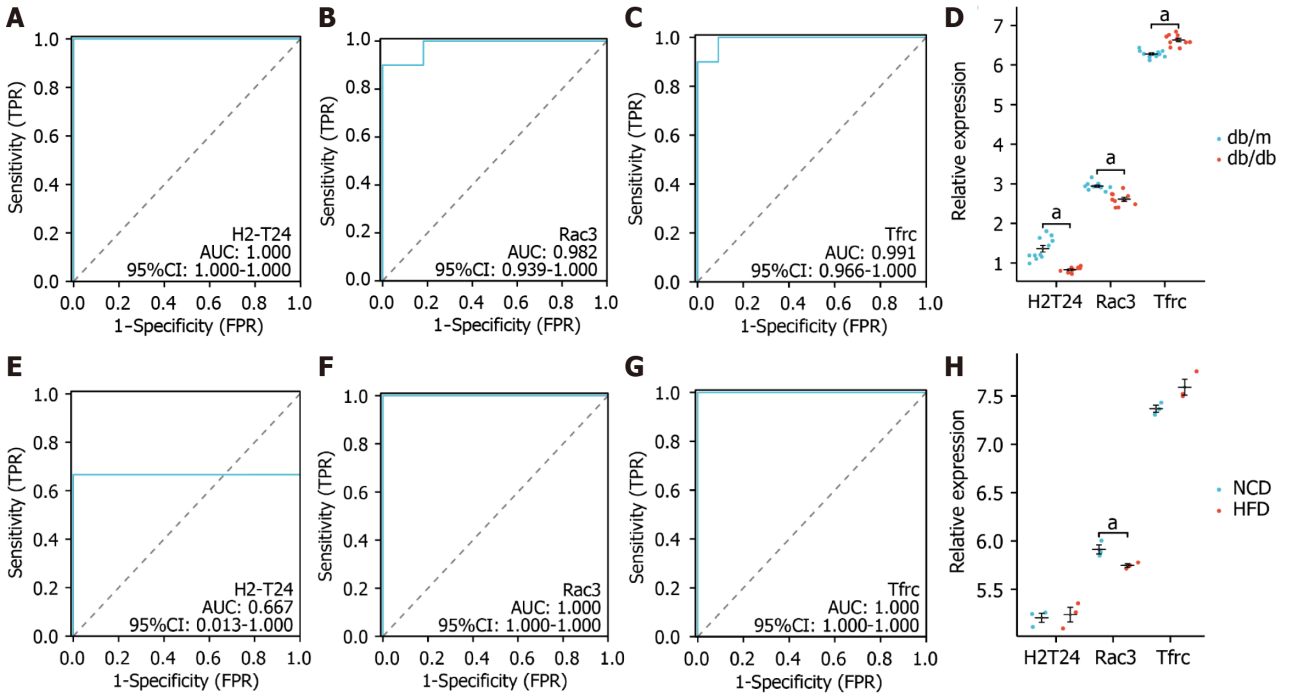


Figure 7 Evaluation of the receiver operating characteristic. A-C: In GSE125387, the receiver operating characteristic (ROC) curve for each candidate gene (*H2-T24*, *Rac3*, and *Tfrc*) was analyzed; D: The levels of expression for the three genes in GSE125387; E-G: In GSE152539, the ROC curve for each candidate gene (*H2-T24*, *Rac3*, and *Tfrc*) is displayed; H: The levels of expression for the three genes in GSE152539 were also confirmed. $^{a}P < 0.05$. AUC: Area under the curve; 95%CI: 95% confidence interval; TPR: True-positive rate; FPR: False-positive rate.

An increasing number of evidence suggests a connection between microglia and cognitive decline. Microglia are the main components of the brain's natural defense system, and play a vital role in neuroinflammation, which is strongly linked to cognitive impairment associated with T2DM[41,42]. Activation of microglia can lead to neuroinflammation and neuronal damage, resulting in cognitive impairment[43]. A review highlighted the complex role of microglia in cognition, including their involvement in neurogenesis, synaptic pruning, and learning and memory. And it may hold potential targets for treating cognitive impairment[44]. A single-cell sequencing study confirmed the opinion that microglia in the hippocampus and immune system play a vital role in diabetes-associated cognitive impairment[45]. Focusing on the immune system and neuroinflammation could offer a promising pathway for creating new treatment approaches to enhance cognitive abilities in T2DM.

Through machine learning, finally, 3 immune-related DEGs have been identified. To verify our findings, we established a mouse model of T2DM and performed quantitative analysis of gene expression, which revealed that 3 genes - *H2-T24*, *Rac3*, and *Tfrc* - showed expression trends consistent with our bioinformatics results.

H2-T24 (Histocompatibility 2, T region locus 24) has been shown to play an important role in immune function. *H2-T24* is associated with the microglia activation after cerebral ischemia[46]. *H2-T24* has a significant impact on the development of AD[47]. *H2-T24* is associated with hippocampus-based memory impairment by endogenous retrovirus[48]. These findings suggest that *H2-T24* is related to neuroinflammation. However, research on *H2-T24* in cognitive impairment is limited. Until now, research on the association between *H2-T24* and diabetes is rare. However, it is worth noting that *H2-T24* is not a homologous gene in humans. Therefore, we decided not to proceed with the drug predictions that were planned.

Rac3, a small GTPase belonging to the Rho family, is primarily found in the brain[49]. Rho family GTPase signaling pathways have been proposed to be linked to diabetes[50]. *Rac* family can inhibit PTEN[51], which is a critical negative regulator in the PI3K pathway of insulin signaling[52,53]. We propose that the *Rac* family has an effect on insulin signaling through inhibiting PTEN, and *Rac3* deficiency affects glucose homeostasis and insulin sensitivity. We consider that insulin resistance is a contributing factor to cognitive dysfunction. *Rac3* plays a critical role in the regulation of dendritic spine development and synaptic plasticity in the hippocampus[54]. The activation of STAT3 and ERK by *Rac3* stimulates the proliferation and migration of glioma cells[55]. *Rac3* is crucial for regulating microglial activation and neuronal inflammation in response to brain injury related to the HMGB1 signaling pathway[56]. In an AD study, the expression of *RAC3* was decreased[57]. Our study's dataset and experimental validation revealed that the expression of *Rac3* was notably reduced in T2DM mice, potentially resulting in cognitive decline. Further research on its functions and potential therapeutical applications in neurodegenerative disorders is needed.

The expression of *Trfc* (transferrin receptor) is significantly increased in neural tissues during neuronal regeneration and repair[58]. The transport of iron across the blood-brain barrier, which is crucial for appropriate neuronal activity, is controlled by *Trfc*[59]. Recent studies have linked *Trfc* to neuroinflammatory conditions. *Tfrc* was upregulated in activated microglia in rats with central pain[60]. *Tfrc* was significantly upregulated in the brains of Parkinson's disease[61]. In AD, *Trfc* is overexpressed, leading to altered iron homeostasis and oxidative stress[62]. The reduction of iron in the brain

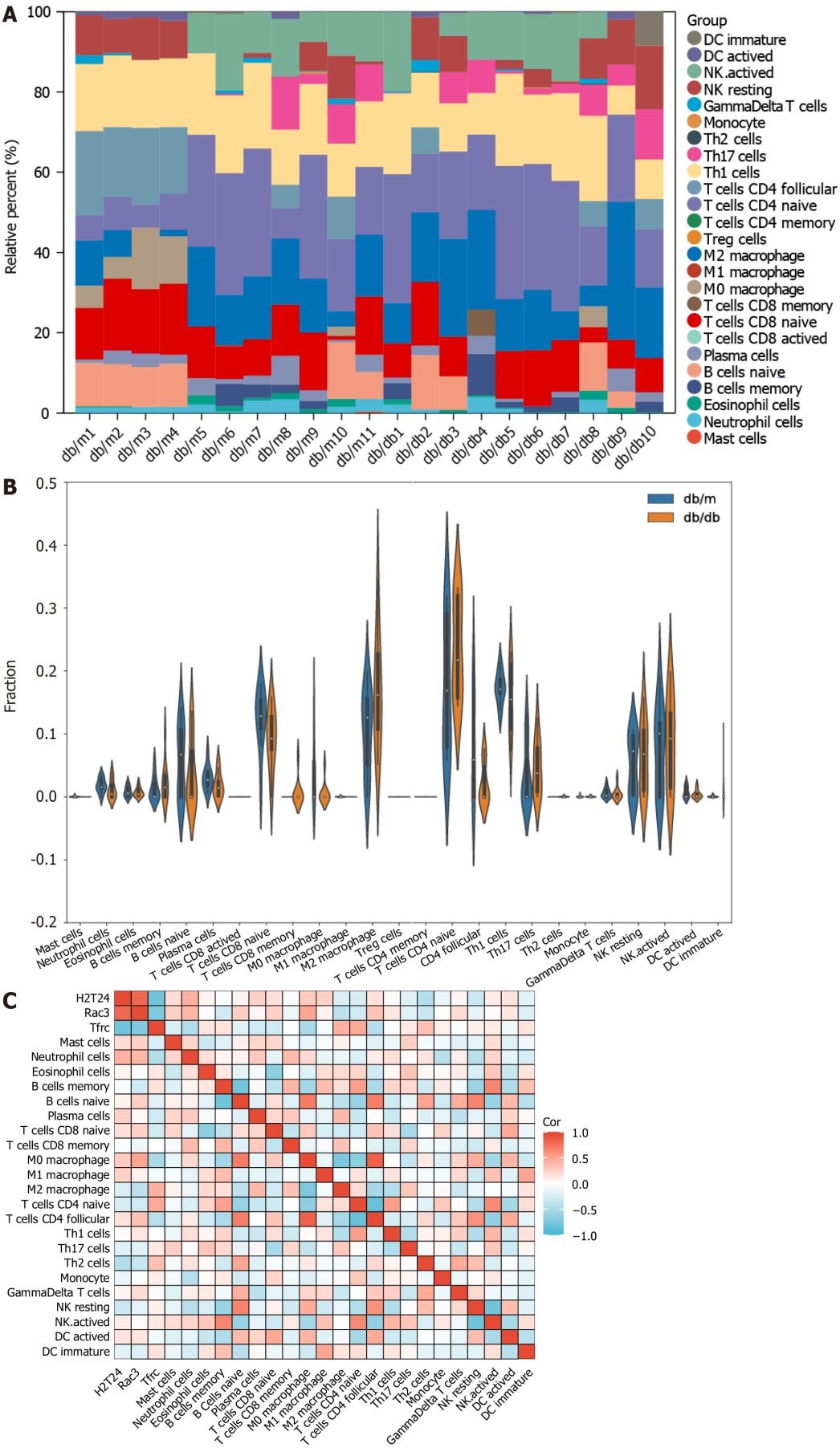


Figure 8 Immune cell infiltration analysis to gain a deeper understanding of the immune regulation involved in the hippocampus of type 2 diabetes mellitus. A: The bar plot visualizes the distribution of 25 different types of immune cells across various samples; B: The violin plot visualizes the

comparison of the proportion of 25 types of immune cells between db/db and db/m; C: The correlation between the compositions of 22 types of immune cells and 3 hub genes. Various immune cells show the infiltration. Cor: Correlation coefficient.

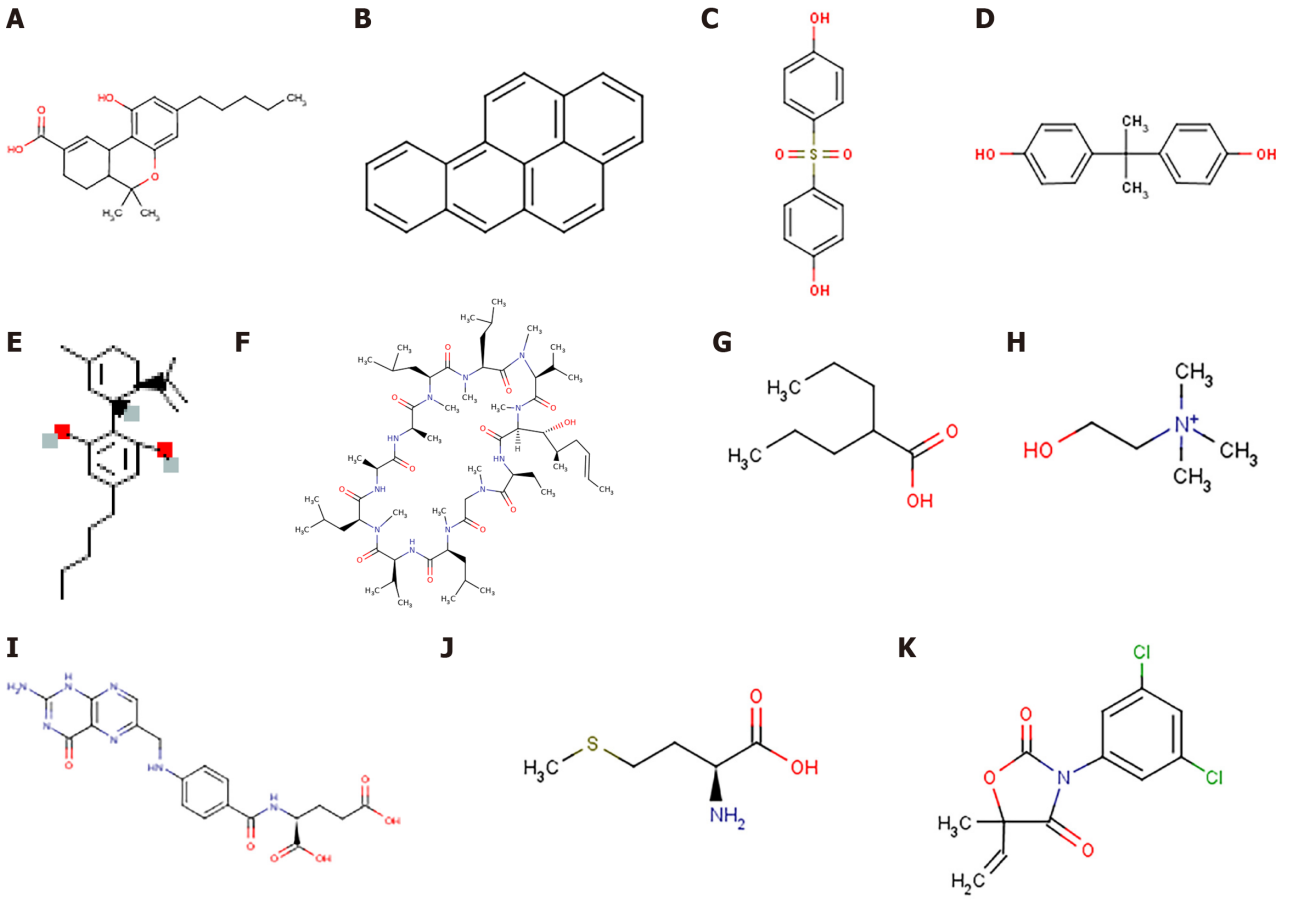


Figure 9 Two-dimensional structures of potential compounds. A: 11-nor-delta(9)-tetrahydro; B: Benzo(a)pyrene; C: Bis(4-hydroxyphenyl)sulf; D: Bisphenol A; E: Cannabinoids; F: Cyclosporine; G: Valproic Acid; H: Choline; I: Folic Acid; J: Methionine; K: Vinclozolin.

presents a new approach to treating AD, indicating that *Tfrc* may serve as a promising target for therapeutic intervention in AD[63]. In another AD study, the expression of *TFRC* was increased[57]. However, there are some conflicting conclusions about *Trfc* in AD. For instance, patients with AD experience a notable reduction in *Tfrc* levels within their peripheral blood mononuclear cells[64]. Hence, further comprehensive and thorough investigations are required to explore the function of *Tfrc* in the nervous system. In another bioinformatics analysis study, *Tfrc* has been described as playing a role in T2DM and neurological diseases[65]. In T2DM, increased iron stores have been found to predict the development of the disease[66]. Circulating *Tfrc* is associated with the relationship among post-load glucose, insulin resistance, and T2DM[67]. We consider that *Tfrc* overexpressed is associated with insulin resistance and thus contributes to cognitive dysfunction. Additional investigation is required to clarify the correlation between *Tfrc* and neuroinflammation, as well as to examine the possibility of *Tfrc* as a target for therapy.

Metabolism and the immunological state are unavoidably intertwined[68]. The metabolic disorders, such as hyperglycemia and hyperlipidemia, induce a state of inflammation in the body in individuals with T2DM. Immune dysregulation is common in cognition impairment. During the study, we employed CIBERSORT to examine the infiltration of immune cells and observed variations in several cell types between the diabetes group and the control group, despite the absence of any significant statistical disparity. This gives us an implication that different immune states affect cognitive function.

Eleven potential drugs were identified in this study. These drugs have a variety of functions, and when the dosage or duration of application is different, it is possible to have the opposite effects on cognitive function. Cyclosporine is an immunosuppressant, mainly used for rejection after organ and tissue transplantation. During surgery under general anesthesia, Cyclosporine treatment can increase ATP levels in the cerebral cortex and improve learning and memory function[69]. Valproic acid is commonly used in the treatment of epilepsy and bipolar affective disorder. It is reported that it improves cognitive function in patients with bipolar affective disorder[70]. However, long-term use of valproic acid can impair cognitive function[71]. Valproic acid exposure can cause autism in prepubertal rats[72]. Choline is a constituent of biological membranes and precursor of acetylcholine in cholinergic neurons. It can promote brain development and improve memory[73]. Lifelong choline supplementation may ameliorate AD by attenuating microglial

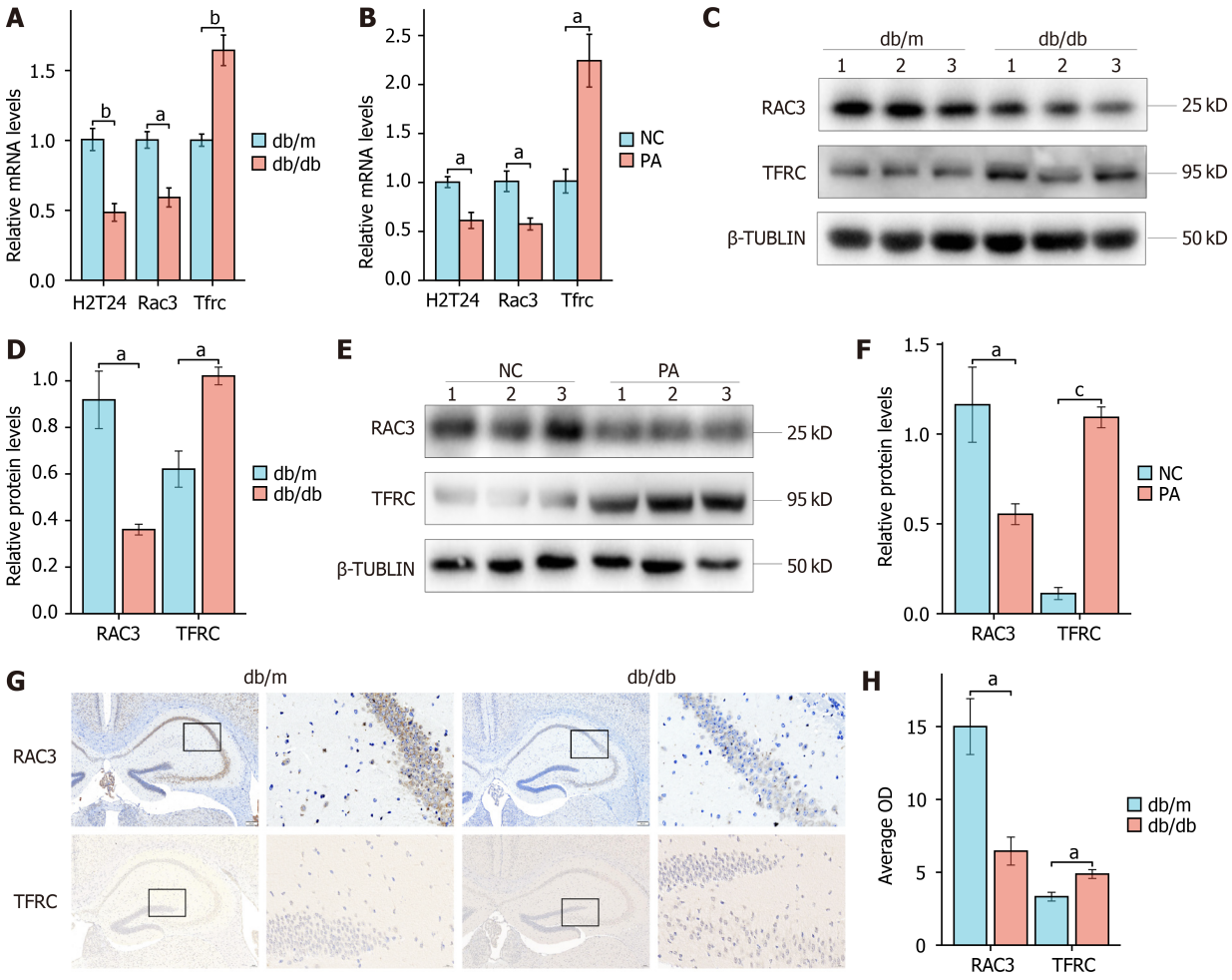


Figure 10 Validation of the expression of hub genes *in vivo* and *in vitro*. A: Hub genes mRNA expression in db/db and db/m mice by reverse transcription quantitative real-time PCR (RT-qPCR). In comparison to db/m mice, *H2-T24* and *Rac3* showed a reduction in expression in db/db mice, whereas *Tfrc* demonstrated an elevation in expression in db/db mice; B: Palmitic acid (PA) was used as a type 2 diabetes mellitus model *in vitro*. Hub genes mRNA expression in PA and negative control (NC) groups by RT-qPCR. *H2-T24* and *Rac3* showed a reduction in expression in the PA group, whereas *Tfrc* demonstrated an elevation in expression in the PA group; C: Protein levels of RAC3 and TFRC in db/db and db/m mice by western blotting. Each lane represents an independent mouse hippocampus; D: Analysis of relative protein levels in db/db and db/m mice. In comparison to db/m mice, *Rac3* showed a reduction in expression in db/db mice, whereas *Tfrc* demonstrated an elevation in expression in db/db mice; E: Protein levels of RAC3 and TFRC in PA and NC groups by western blotting. Each lane represents an independent experiment of cell lysate; F: Analysis of relative protein levels in PA and NC group. *Rac3* showed a reduction in expression in the PA group, whereas *Tfrc* demonstrated an elevation in expression in the PA group; G: Protein expression of RAC3 and TFRC in db/db and db/m mice by immunohistochemistry; H: Analysis of average OD in db/db and db/m mice. In comparison to db/m mice, *Rac3* showed a reduction in expression in db/db mice, whereas *Tfrc* demonstrated an elevation in expression in db/db mice. Data are expressed as the mean ± SEM (n = 3). $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. NC: Negative control; PA: Palmitic acid.

activation[74]. It is well known that folic acid is closely related to fetal neurodevelopment. The deficiency of folic acid can result in elevated levels of homocysteine, thereby contributing to the development of atherosclerosis, stroke, diabetes, and other related conditions[75]. Folic acid supplementation affects cognition and inflammation in patients with AD[76]. A Methionine-restricted diet can improve cognitive function[77]. Another study of Chinese adults revealed that animal methionine and plant methionine intake were positively and inversely associated with cognition[78]. These drugs, which have beneficial effects on the nervous system, may become therapeutic options for diabetes-related cognitive impairment. However, 11-nor-delta (9)-tetrahydrocannabinol-9-carboxylic acid[79], Cannabinoids[80], Benzo(a)pyrene[81], Bis(4-hydroxyphenyl)s, Bisphenol A[82] and Vinclozolin[83] are neurotoxic and can lead to cognitive impairment. This suggests that patients with diabetic cognitive impairment should avoid exposure to neurotoxic drugs.

Our study has certain restrictions. Firstly, given the scarcity of hippocampus datasets from diabetic mice with validated cognitive impairment, we used only one dataset for bioinformatics analysis and the other for validation of hub genes. Secondly, our validation of the hub genes has been limited to diabetic mice, and we do not have the backing of clinical data. Furthermore, despite performing a thorough bioinformatics analysis in this study, we did not carry out additional experiments to validate the impact of immune-related genes on cognitive function. Therefore, additional investigation is required to further explore the precise mechanism of immunometabolism regulation in diabetic cognitive impairment both *in vivo* and *in vitro*. Our future investigation will concentrate on this new course.

CONCLUSION

In summary, we identified the differences in immune-related genes in the hippocampus between T2DM and control mice by comprehensive bioinformatics analysis. The immune-related DEGs were closely related to microglia. 3 hub genes were screened and verified- *H2-T24*, *Rac3*, and *Tfrc*. They were associated with a variety of immune cells. We verified the expression of these 3 genes *in vivo* and *in vitro*, consistent with the bioinformatics analysis. 11 drugs associated with *RAC3* and *TFRC* were identified. These findings suggest that they are co-regulatory molecules of immunometabolism in diabetic cognitive impairment, and provide a new insight in the treatment of diabetic cognitive impairment.

ARTICLE HIGHLIGHTS

Research background

Cognitive decline in type 2 diabetes mellitus (T2DM) is a complex and progressive condition that demands additional research for complete understanding. Neuroinflammation is seen as a primary mechanism, with the immune system significantly influencing the disease's advancement.

Research motivation

Cognitive impairment in T2DM is complex and evolving, necessitating deeper research. The immune system significantly impacts its progression.

Research objectives

To pinpoint and confirm hippocampus immune-related genes linked to cognitive impairment in T2DM.

Research methods

Using the Gene Expression Omnibus database GSE125387, we pinpointed genes differentially expressed between T2DM and control mice, and identified key module genes related to T2DM through Weighted Gene Co-Expression Network Analysis. We conducted Gene Set Enrichment Analysis for these genes and built a protein-protein interaction network, employing Lasso regression and Random Forest to identify three hub genes. These genes underwent immune cell infiltration analysis and were validated in GSE152539 using receiver operating characteristic curve analysis. Validation included RT-qPCR, Western blotting, and immunohistochemistry at mRNA and protein levels, both *in vivo* and *in vitro*. Furthermore, we discovered 11 potential drugs linked to these genes using the Comparative Toxicogenomics Database.

Research results

We identified 576 DEGs from GSE125387 and intersected them with T2DM module and immune-related genes, finding 59 immune system-related genes. Machine learning pinpointed three hub genes (*H2-T24*, *Rac3*, *Tfrc*), linked to various immune cells. These genes were validated in GSE152539, with experiments at mRNA and protein levels *in vivo* and *in vitro*, aligning with our bioinformatics analysis. Additionally, 11 potential drugs related to *RAC3* and *TFRC* were identified using the Comparative Toxicogenomics Database.

Research conclusions

The immune system plays a significant role in cognitive impairment in T2DM. The immune-related differently expressed genes in hippocampus were closely related to microglia. We confirmed the expression of three such genes both *in vivo* and *in vitro*, in line with our bioinformatics findings. Three hub genes screened were associated with a variety of immune cells. Moreover, 11 drugs related to *RAC3* and *TFRC* were identified.

Research perspectives

These genes are as co-regulatory molecules in the immunometabolism of diabetic cognitive impairment, offering new perspectives for its treatment.

FOOTNOTES

Author contributions: The design of this study was carried out by Hou XG; the collection and analysis of bioinformatics data, experimental validation, and writing of the manuscript were carried out by Gao J; Zou Y took on the task of conducting statistical analysis on the experimental data; Lv XY was responsible for animal modeling; Chen L contributed to the literature research; the final manuscript was read and approved by all the authors.

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ORIGINAL ARTICLE

Basic Study

Long-term effects of gestational diabetes mellitus on the pancreas of female mouse offspring

Enriqueta Muñoz-Islas, Edgar David Santiago-SanMartin, Eduardo Mendoza-Sánchez, Héctor Fabián Torres-Rodríguez, Laura Yanneth Ramírez-Quintanilla, Christopher Michael Peters, Juan Miguel Jiménez-Andrade

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Abstract

BACKGROUND

Prolonged fetal exposure to hyperglycemia may increase the risk of developing abnormal glucose metabolism and type-2 diabetes during childhood, adolescence, and adulthood; however, the mechanisms by which gestational diabetes mellitus (GDM) predisposes offspring to metabolic disorders remain unknown.

AIM

To quantify the nerve axons, macrophages, and vasculature in the pancreas from adult offspring born from mouse dams with GDM.

METHODS

GDM was induced by *i.p.* administration of streptozotocin (STZ) in ICR mouse dams. At 12 wk old, fasting blood glucose levels were determined in offspring. At 15 wk old, female offspring born from dams with and without GDM were sacrificed and pancreata were processed for immunohistochemistry. We quantified the density of sensory [calcitonin gene-related peptide (CGRP)] and tyrosine hydroxylase (TH) axons, blood vessels (endomucin), and macro-phages (CD68) in the splenic pancreas using confocal microscopy.

RESULTS

Offspring mice born from STZ-treated dams had similar body weight and blood glucose values compared to offspring born from vehicle-treated dams. However, the density of $CGRP^+$ and TH^+ axons, $endomucin^+$ blood vessels, and $CD68^+$ macrophages in the exocrine pancreas was significantly greater in offspring from

mothers with GDM *vs* control offspring. Likewise, the microvasculature in the islets was significantly greater, but not the number of macrophages within the islets of offspring born from dams with GDM compared to control mice.

CONCLUSION
GDM induces neuronal, vascular, and inflammatory changes in the pancreas of adult progeny, which may partially explain the higher propensity for offspring of mothers with GDM to develop metabolic diseases.

Key Words: Gestational diabetes mellitus; Immunohistochemistry; Confocal microscopy; Pancreas; Offspring

Core Tip: Gestational diabetes mellitus (GDM) predisposes offspring to develop metabolic disorders later in life, however, the underlying mechanisms are unknown. Here, using a well-established model of GDM, we report that while GDM did not modify body weight or blood glucose, it significantly increased the density of nerve axons, blood vessels, and macrophages in the pancreas of adult offspring born from dams with GDM.

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INTRODUCTION

Gestational diabetes mellitus (GDM) is one of the most common complications associated with pregnancy and is defined as any degree of glucose intolerance that occurs with the onset or during pregnancy[1]. The prevalence of GDM has increased substantially in the last few decades, occurring in up to 25% of pregnancies in certain populations[2]. GDM not only results in negative effects on mothers but also on their offspring[1,3]. Epidemiological studies show long-term neurological, cardiovascular, and endocrinological complications in offspring[4-6]. Prolonged fetal exposure to hyperglycemia may increase the risk of developing abnormal glucose metabolism and type-2 diabetes (T2D) during childhood, adolescence, and adulthood[6-9]. However, the mechanisms that contribute to the development of metabolic disorders following maternal hyperglycemia remain elusive.

Recently, there has been considerable scientific interest in understanding the role of pancreatic innervation in regulating glucose metabolism[10]. Sympathetic and sensory nerve fibers innervate the exocrine and endocrine pancreas [11-14]. In experimental diabetes, increased sympathetic innervation of islets has been shown to convey inhibitory signals reducing insulin production and release[15,16]. Clinically, sympathetic hyperactivity precedes the development of diabetes in young non-obese Japanese[17] and Korean[18] adults. Similarly, sensory neuron innervation is also associated with the regulation of glucose metabolism in experimental diabetes. In rats with spontaneous non-insulin-dependent diabetes, there is an increased density of sensory nerve fibers innervating the pancreas that occurs before the development of hyperglycemia[19]. Furthermore, several studies demonstrate an inhibitory influence of sensory neuron-derived neuropeptides on insulin production and sensitivity[20,21].

Previous reports have shown that patients with T2D have increased density of blood vessels surrounding islets compared to healthy individuals[22]. Additionally, in rodent models of spontaneous diabetes, several cellular and vascular abnormalities occur in pancreatic tissue at pre-diabetic stages. Enhanced vascular endothelial growth factor (VEGF) levels have been reported to occur in β-cells leading to disorganized, hypervascularized, and fibrotic islets, progressive macrophage infiltration, and increased proinflammatory cytokine production[23,24]. Altogether, these findings suggest that pathological changes might occur in pancreatic tissue at prediabetic stages even prior to quantifiable hyperglycemia and/or insulin resistance.

Adult mouse offspring born from dams with GDM are more susceptible to developing metabolic disorders[25,26]; however, the underlying pathogenic mechanisms are not fully known. The current study aims to assess whether there are changes in the sensory and sympathetic innervation, vascularization, and macrophage infiltration in pancreatic tissue from adult mouse offspring born from dams with streptozotocin (STZ)-induced GDM.

MATERIALS AND METHODS

Animals

Male (n = 10) and female ICR (n = 20) mice were purchased from Bioinvert Laboratories (Mexico City, Mexico) at an age of 10-12 wk (body weight 20-25 g). They were given one week to acclimate before use. These animals were used for

mating and posterior female offspring mice and all of them were housed at a temperature of 22 °C ± 2 °C, maintained on a 12:12 h light/dark cycle, with free access to food and water following the Norma Oficial Mexicana NOM-062-ZOO-1999. All animal experiments were conducted following the national guidelines and the relevant national laws on the protection of animals. Efforts were made to minimize the number of animals used.

Gestational diabetes model

Mice were mated overnight, two or three females per male. The presence of a vaginal plug the next morning indicated gestation day 0.5 (GD 0.5). Gestational diabetes was induced by intraperitoneal administration of STZ (catalog number S0130; Sigma-Aldrich Co.) dissolved in sodium citrate buffer (pH: 4.5 0.1 M). The STZ was administrated for three consecutive days as follows: 100 mg/kg on gestational day 6.5, 100 mg/kg on gestational day 7.5, and 80 mg/kg on gestational day 8.5[27]. Other pregnant mice were administered sodium citrate buffer (pH: 4.5, 0.1 M) on the same days of gestation (vehicle group, VEH). Dams were randomly allocated to receive STZ or vehicle (Figure 1A). Dams treated with STZ were considered to experience GDM when glucose concentrations were higher than 11.1 mmol/L[28,29]. Based on this criterion, the GDM model was confirmed through the determination of blood glucose of the dams 48 h after the last STZ or VEH administration (at 10.5 d of gestation). The concentration of blood glucose through the caudal vein was measured (mmol/L; Accutrend Plus, Roche) following a 6-h fasting period (Figure 1A). In each offspring group, mice were born from at least 4 different dams. The offspring were weaned at 20-21 d old. Due to our previous study showing no differences in blood glucose concentrations and oral glucose tolerance test between male and female offspring at 14-16 wk old[30], only female offspring were used in this study.

Tissue harvesting and immunohistochemistry

Female mice were humanely sacrificed at 15 wk of age, through deep anesthesia with a mixture of ketamine and xylazine (100/10 mg/kg) followed by transcardiac perfusion, first with phosphate-buffered saline (PBS, 0.01 M, pH: 7.4, 4 °C) and followed by 4% paraformaldehyde in PBS (Figure 1A). The pancreas was dissected from the peritoneal visceral, leaving the spleen and duodenum associated as anatomical reference[31]. Following dissection, tissue specimens were post-fixed for 24 h in the same fixative at 4 °C. Then, the tissue specimens were cryoprotected in 30% sucrose solution at 4 °C for at least 48 h before the tissue was processed for IHC. The pancreata were embedded in Tissue Plus® (catalog Fisher HealthCare, Houston, TX, United States) and cut on a cryostat (Leica CM1900, Leica Biosystems, II, United States) at a thickness of 40 µm in the frontal plane and mounted on glass microscope slides (Superfrost Plus, Fisher Scientific). The pancreatic sections were incubated for 12 h at room temperature with primary antibody against calcitonin gene-related peptide (CGRP, polyclonal rabbit anti-mouse 1:3000; Sigma Aldrich; catalog number C8198) to label primary afferent sensory peptidergic nerve fibers. Sympathetic nerve fibers were labeled with primary antibody against tyrosine hydroxylase (TH, polyclonal rabbit anti-mouse 1:1000; EMD Millipore; catalog number AB152). An anti-endomucin (monoclonal rat anti-mouse 1:500; Santa Cruz; Catalog Number SC-65495) antibody was used for blood vessels. For activated macrophages, an antibody against CD68 primary antibody (Macrosialin; monoclonal rat anti-mouse 1:3000; Bio-Rad; catalog number MCA1957) was employed. Subsequently, preparations were washed in PBS and then incubated for 3 h with the secondary antibody (Cy3 monoclonal donkey anti-rabbit 1:600; Jackson ImmunoResearch; Catalog number 711-165-152 and Cy2 monoclonal donkey anti-rat 1:300; Jackson ImmunoResearch; Catalog number 712-225-150). Then, pancreatic sections were washed in PBS, dehydrated through an alcohol gradient (70%, 80%, 90%, and 100%), cleared in xylene, and coverslipped with a DPX mounting medium. Nuclear stain 4,6-diamidino-2-phenylindole (DAPI; 1:20000; Invitrogen; catalog number D21490) was used to visualize all cell nuclei.

Quantification of the density of nerve fibers, blood vessels, and macrophages

Previous studies have shown that the density of islets of Langerhans[32], as well as the density of sympathetic nerve fibers[31], is not significantly different between the duodenal and splenic pancreatic regions. Thus, in the present study, we determined the density of nerve fibers, blood vessels, and macrophages only in the splenic pancreas (Figure 1D). For this purpose, approximately 12 separate 40-µm-thick frozen sections were obtained from the pancreas of each mouse. To quantify nerve fibers (CGRP and TH), one image per section was acquired within the acinar tissue in the splenic pancreas through a Carl Zeiss scanning confocal laser microscope (Model LSM 800, Jena Germany) using a 20x air, scan zoom 2.0 objective. Then, images were analyzed using ImageJ software (National Institutes of Health) and nerve fibers were manually traced by a blinded investigator, using the freehand line tool, to determine the total length of nerve fibers. Data from at least three sections per mouse (separated by at least 100 µm) from the splenic pancreas were recorded and averaged. The total volume was calculated by tracing the area of the image and multiplying it by the thickness of the section (40 µm). The signal/noise ratio for nerve profiles within the islets was very low which did not allow us to obtain reliable quantification. Therefore, the density of nerve fibers was performed only in the exocrine pancreas. Data were expressed as the total length of nerve fibers per volume of the acinar pancreatic tissue (mm/mm^3).

The quantification of the density of CD68-immunoreactive cells in pancreatic tissue was adapted from previous studies [33-35]. After IHC staining, three confocal images were acquired with a 20x air objective (aperture of 0.5; scan zoom 2.0 ×). Areas with greater CD68$^+$ expression were identified in the acinar cells or the islets of Langerhans of the splenic pancreas located 3 mm away from the spleen. Quantification was performed by visualizing all focal planes of the Z-axis counting as positive those CD68$^+$ profiles having one DAPI-stained nucleus. The number of cells was calculated in 1mm^3 of volume. For pancreas islets, it was considered the area of the islet.

The quantification of the density of endomucin-immunoreactive blood vessels was made both in acinar cells and islets of Langerhans (Figure 1D). One image per section was acquired within the acinar cells in the splenic pancreas with an epifluorescence microscope (Axio Scope.A1, Carl Zeiss, Jena, Germany; 20x), or in the islets of the pancreas with a

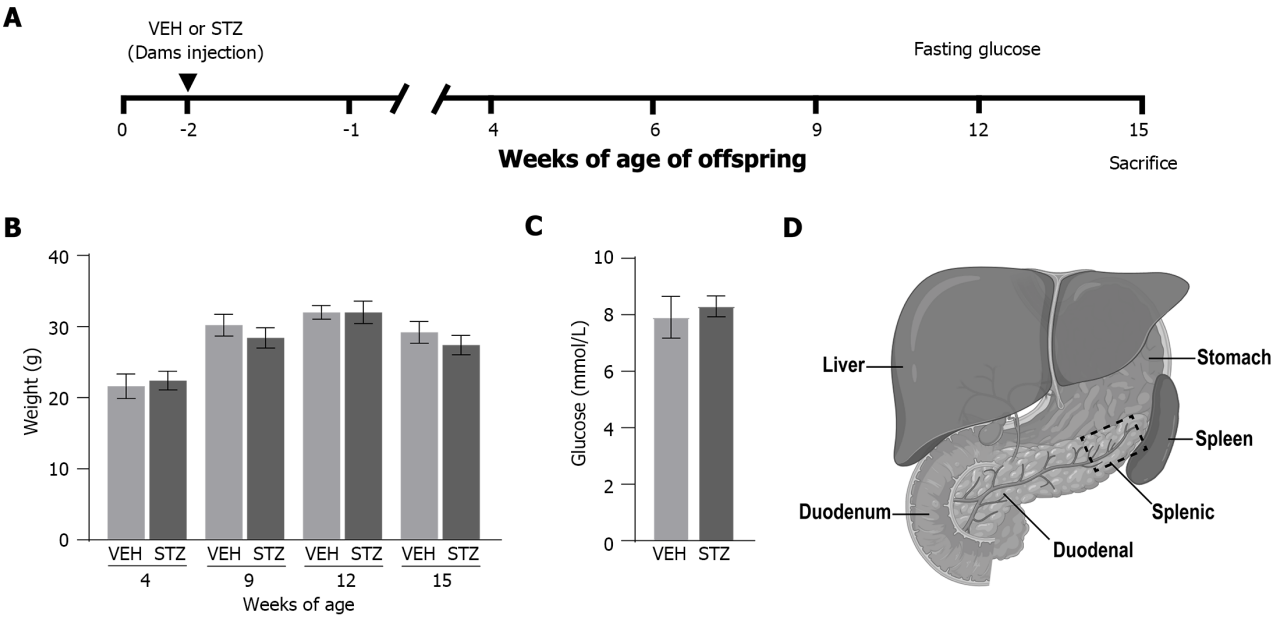

Figure 1 Experimental design, body weight, and blood glucose levels of female offspring born from control dams or dams with gestational diabetes mellitus. A: Diagram showing the experimental design and timeline; B and C: Female offspring born from dams treated with streptozotocin during pregnancy did not show significant changes in body weight or blood glucose compared to offspring born from dams treated with vehicle as controls; D: Scheme showing the region selected for quantification (splenic pancreas; square) of the neuronal, vascular, and inflammatory changes using immunohistochemistry and confocal microscopy. Data are shown as mean ± SEM, *n* = 5 per group. STZ: Streptozotocin; VEH: Vehicle.

confocal microscope. The number of blood vessels was determined in three different sections of the splenic pancreas. A blood vessel was counted only when it had a diameter between 2-10 μm[35]. The results were expressed as the mean number of blood vessels per area of interest (mm^2). The area was obtained for acinar cells tracing the image, and for islets, it was adjusted per total islet area.

Immunohistochemistry on whole pancreatic tissue

This clearing method was adapted from a previous study[36]. Briefly, intestines and mesenteric fat tissues were carefully dissected at the level of the pancreas and duodenum. Intestines were flushed with PBS 0.01 M using a 23G syringe to remove fecal content. The tissues were fixed with 4% paraformaldehyde at 4 °C for 24 h and then washed with PBS 0.01 M three times for 1 h each. The samples were dehydrated with gradients of ethanol (20%, 40%, 60%, and 80%) and then incubated at 4 °C overnight with a solution of 30% H_2O_2 and 100% ethanol (1:10) to decolorize. The tissues were rehydrated through reverse ethanol gradients for 30 min each (100%, 80%, 60%, 40%, and 20%) and 0.01M PBS for 1 h twice. The samples were permeabilized with a solution containing 0.2% Triton X-100 (Sigma Aldrich, Catalog Number X-100), 0.1% Deoxycholate sodium (Sigma Aldrich, Catalog Number D6750-25G), 10% DMSO (Fisher Bioreagents, Catalog Number BP231-1), in 0.01 M PBS (solution pH: 6.5) and incubated for 72 h at 37 °C. Subsequently, the samples were blocked with 0.2% Triton X-100/10% DMSO/5% Normal Donkey Serum/10 mg/mL heparin (Sigma Aldrich, Catalog Number H3393-100KU) in 0.01M PBS solution for 24 h (solution pH: 6.5). Then the samples were stained with primary antibody against endomucin and secondary antibody (Cy3 monoclonal donkey anti-rat 1:600) for 72 h each at room temperature. Later, whole pancreatic tissue was washed with 0.01 M PBS and dehydrated with gradients of ethanol. Finally, the samples were mounted in the histology chamber, and cleared in dibenzyl ether (Sigma Aldrich; Catalog number 108014-1KG). Three-dimensional imaging of islets of Langerhans was obtained using a ZEISS LSM 800 confocal microscope with a 20x air objective. Imaris Viewer Version 10.0.1 software package for Windows (Oxford Instruments Inc.) was used to generate 3D images.

Statistical analysis

Data were represented as the mean ± SEM of groups of 5 mice each. Either a student *t*-test or two-way repeated measures ANOVA followed by a Tukey *post hoc* test was run when appropriate. Statistical significance was accepted at $P < 0.05$. All statistical analyses were performed using GraphPad Prism version 8.0 software package for Windows (GraphPad Software Inc., San Diego, CA, United States).

RESULTS

Effects of STZ-induced GDM on blood glucose levels and body weight of adult mice offspring

The administration of STZ in dams resulted in fasting blood glucose levels of (13.26 mmol/L ± 1.18 mmol/L), which were

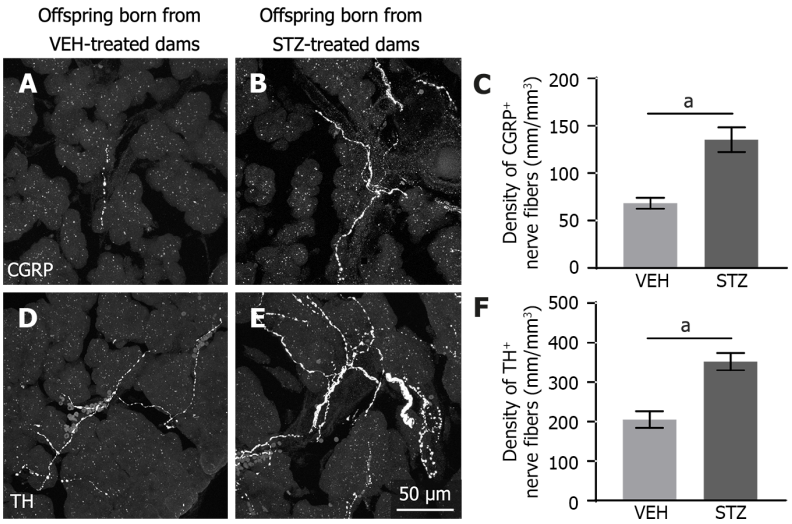

Figure 2 Adult female offspring born from dams with streptozotocin-induced gestational diabetes mellitus have increased density of sensory and sympathetic nerve fibers innervating the pancreatic acinar cells. A and B: Representative confocal images of calcitonin gene-related peptide (CGRP) expressing small diameter peptidergic sensory nerve fibers; C and F: Significantly greater densities of CGRP$^+$ and tyrosine hydroxylase$^+$ (TH$^+$) nerve fibers were found in offspring born from dams with GMD as compared to control offspring; D and E: Enzyme TH expressing postganglionic sympathetic nerve fibers in acinar pancreatic tissue (40 μm-thick) from offspring born of vehicle or streptozotocin injected dams; $^aP < 0.05$ Student *t* test, $n = 5$ per group. Data are shown as mean ± SEM. CGRP: Calcitonin gene-related peptide; TH: Tyrosine hydroxylase; VEH: Vehicle; STZ: Streptozotocin.

significantly higher as compared to those dams treated with vehicle (9.57 mmol/L ± 0.26 mmol/L). To determine the influence of GDM on the development of offspring, body weight was registered every week, and fasting blood glucose levels were determined at week 12 of age (Figure 1A). There were no significant changes in body weight (Figure 1B) or blood glucose levels (Figure 1C) of offspring born from dams treated with STZ compared to offspring born from dams treated with vehicle.

STZ-induced GDM increased the density of sensory and sympathetic nerve fibers innervating the acinar pancreatic tissue from adult female offspring

Sensory and sympathetic nerve fibers innervating the acinar cells from the splenic pancreas were detected using CGRP and TH antibodies, respectively. Representative confocal images show CGRP- (Figure 2A and B) and TH- (Figure 2D and E) immunoreactive nerve fibers present along the acinar cells. CGRP nerve fibers (Figure 2A) were less prominent than TH nerve fibers (Figure 2D) in control tissue. However, exocrine pancreatic tissue from adult offspring born from mice treated with STZ showed a significantly greater innervation of sensory CGRP (Figure 2B and C), and sympathetic TH (Figure 2E and F) fibers as compared to control mice (Figure 2A and D).

GDM increases blood vessel density both in acinar cells and islets of Langerhans

Endomucin-positive blood vessels were distributed throughout acinar tissue and had a tortuous appearance (Figure 3A and B). Quantitative analysis using confocal images showed that the density of blood vessels in offspring born from dams treated with STZ (Figure 3B and C) was significantly higher compared with offspring treated with vehicle (Figure 3A).

Endomucin immunoreactivity showed blood vessels well distributed through the pancreatic islet. These endomucin$^+$ blood vessels had a tortuous form. They were in a greater number in the tissue of offspring born from STZ-treated dams (Figure 3E) as compared to the control group (Figure 3D). The quantitative analysis revealed that GDM induced a significant increase in the number of blood vessels per total islet area in offspring from GDM dams as compared with offspring from dams treated with vehicle (Figure 3F). The islet area in offspring born from control dams was 124284.25 μm^2 ± 992.40 μm^2 and this tends to decrease to 91431.23 μm^2 ± 1382.00 μm^2 in progeny from dams with STZ-induced GDM ($P = 0.355$; unpaired *t*-test). Additionally, a solvent-based clearing method from whole pancreatic tissue was used to visualize the distribution of the blood vessels in an intact whole islet of Langerhans. Three-dimensional imaging of the islet showed increased intra-islet vasculature in offspring from GDM dams *vs* offspring from control dams (Figure 3G and H).

STZ-induced GDM increased the density of macrophages in the exocrine, but not the endocrine pancreatic tissue of adult female offspring

Immunohistochemical and confocal analysis revealed that macrophages are uniformly distributed in the acinar cells and pancreatic islets. In the acinar cells, the CD68$^+$ macrophages in the control tissue (Figure 4A) had a smaller size compared to those in the pancreatic tissue of adult offspring born from dams with GDM (Figure 4B). Macrophages in the acinar cells of GDM offspring also had a hypertrophied multivacuolated appearance indicative of active phagocytic macrophages (Figure 4B). GDM induced a statistically significant increase in the number of macrophages in the acinar pancreatic tissue of the offspring as compared to the control group (Figure 4C). On the other hand, quantitative analysis using confocal

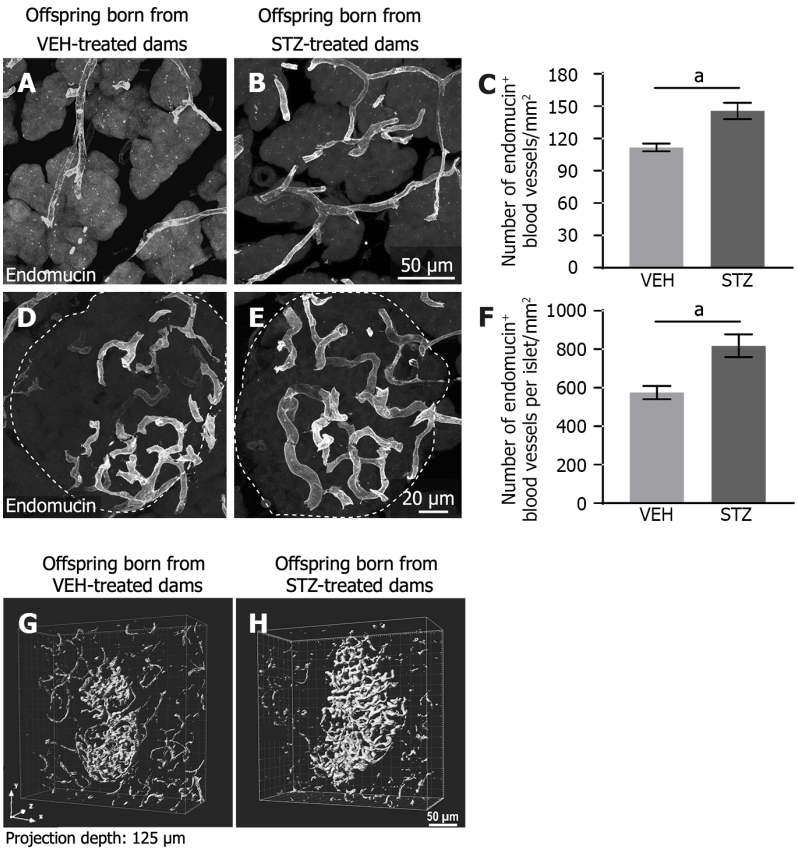

Figure 3 Adult female offspring born from dams with streptozotocin-induced gestational diabetes mellitus have a greater density of blood vessels vascularizing the acinar cells and the pancreatic islets. A, B, D and E: Representative confocal images of vehicle showing the endocrine and exocrine pancreatic tissue immunostained for endomucin blood vessels from adult progeny born from dams treated with vehicle or streptozotocin (STZ); C and F: There was a significantly greater density of blood vessels positive to endomucin in the acinar cells and the islets of Langerhans of offspring mice born from mice treated with STZ as compared to control mice; G and H: 3D visualization of the islet of Langerhans from whole pancreatic tissue processed with a solvent-based clearing method. Three-dimensional images of a cleared pancreas show an increased intra-islet vasculature. $^{a}P < 0.05$ Student *t* test, *n* = 4-5 per group. Data are shown as mean ± SEM. VEH: Vehicle; STZ: Streptozotocin.

images shows that macrophages in the pancreatic islets were distributed at the same proportions in the control group (Figure 4D and F) compared to the STZ group (Figure 4E).

DISCUSSION

This study reports for the first time that while adult offspring born from STZ-induced GDM do not exhibit alterations in body weight and glucose levels as compared to their age-matched respective controls[30], they have significant alterations at a cellular level in their pancreas. These include an increased density of a subset of sensory and sympathetic nerve fibers innervating exocrine pancreatic tissue along with an enhanced vasculature and macrophage infiltration in the exocrine pancreas. It was also found that there is an increased vasculature within the islets in these adult offspring exposed to GDM.

Both in the present study and in our previous report[30], we found that levels of blood glucose and glucose tolerance in adult mouse offspring (15 wk old) were not significantly impaired. Accordingly, adult mouse offspring born from dams with STZ-induced GDM at 3-6 months of age had normal blood glucose levels and did not exhibit any glucose intolerance [37]. However, it is possible to hypothesize that GDM-induced fetal hyperglycemia could program the body to respond differently to metabolic challenges later in life, increasing the susceptibility to develop metabolic alterations and dysfunction in glucose metabolism when they are older. In support of this, a recent study showed that while mice offspring born from dams with GDM are normoglycemic, they are more susceptible to becoming glucose intolerant when they are exposed to a short period of a high-fat diet[25]. Additionally, mouse offspring born from STZ-induced GDM dams develop both impaired glucose tolerance and decreased insulin sensitivity when they are 40 wk, but not, at 12 wk old[26].

It has been reported that the risk of prediabetes/diabetes in the offspring of mothers with GDM is eight times greater compared to offspring from mothers without GDM[6-9]. Given that the risk of developing T2D is greater in individuals whose mothers were diabetic when they were in utero as compared with offspring born from diabetic fathers and siblings born before the onset of maternal diabetes, it has been proposed that these long-term consequences of altered glucose

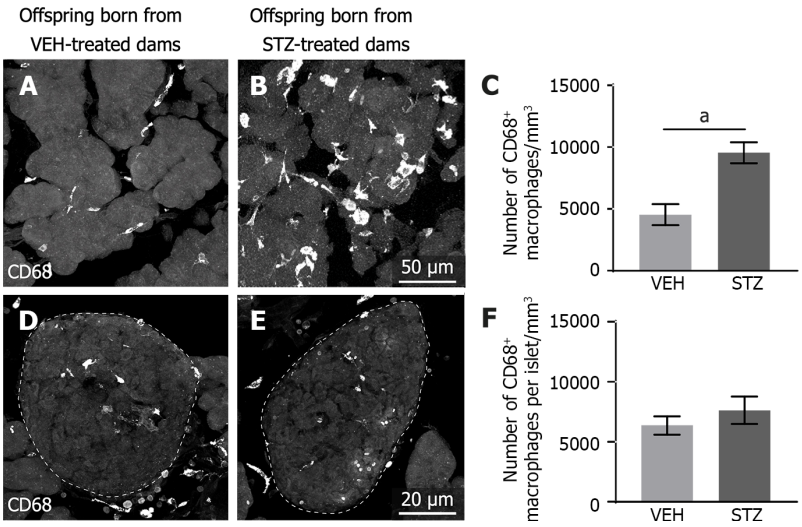

Figure 4 Adult female offspring born from dams with streptozotocin-induced gestational diabetes mellitus have an increased number of macrophages in the pancreatic acinar cells, but not in the pancreatic islets. A, B, D, and E: Representative confocal images of pancreatic cells expressing CD68 (cluster of differentiation 68) for macrophage staining in acinar pancreatic tissue or pancreatic islets from offspring born of vehicle or streptozotocin (STZ) injected dams (40 μm-thick); C and F: Significantly greater number of macrophages were found in the acinar pancreatic tissue, but not in the pancreatic islets of offspring born from dams treated with STZ compared to control mice. $^{a}P < 0.05$ Student *t* test, $n = 4$-5 per group. Data are shown as mean ± SEM. CD68: Cluster of differentiation 68; VEH: Vehicle; STZ: Streptozotocin.

metabolism result mainly from the fetal environment in GDM[38]. Intrauterine exposure to hyperglycemia may generate an *in-utero* environment around the fetus which programs them to disease during adulthood. This phenomenon has been recently called "*metabolic memory*"[39]. However, the specific underlying mechanisms by which fetal exposure to hyperglycemia may affect fetal development and impair glucose metabolism in the offspring are not fully known. It was recently demonstrated that adult mouse offspring born from STZ-induced GDM have dyslipidemia, insulin resistance, and glucose intolerance with advanced age (at 40 and 70, but not at 12 wk). Additionally, metabolic dysfunction and alterations in patterns of DNA methylation of genes involved in regulating glucose metabolism have also been reported [26]. Consistent with these studies, we also report in the current study GMD-induced long-term abnormalities and pathological changes in the pancreatic tissue of mouse offspring.

An increased density of TH nerve fibers in the exocrine pancreas has been reported in mice that spontaneously develop T2D[15] and in patients with T2D[10]. Moreover, the density of these sympathetic nerve fibers in pancreatic tissue parallel worsening glucose tolerance[10]. Finally, it has been shown that sympathetic nerve hyperactivity precedes hyperinsulinemia in young nonobese Japanese individuals[17], and a deviation in sympathovagal imbalance to sympathetic activity precedes the development of diabetes in young Korean adults[18]. Regarding CGRP nerve fibers, both exocrine and endocrine pancreata from mice[31] and different mammals including humans[40] are highly innervated by this subtype of sensory nerve fiber. Chemical ablation of CGRP nerve fibers prevents glucose homeostasis deterioration through increased insulin secretion in Zucker diabetic rats, an animal model for some aspects of human T2D[41].

Furthermore, a significant increase in the length of CGRP nerve fibers innervating pancreatic tissue from Otsuka Long-Evans Tokushima fatty rats (a model of human non-insulin-dependent diabetes) at 16 wk old was previously reported, even though they did not have increased fasting plasma glucose levels[19]. Preclinical and clinical evidence has also shown an increased vascularization, altered microvasculature, and macrophage infiltration in pancreatic tissue, and it has been proposed that these anatomical changes may contribute to a deterioration of -cell/islet function and exacerbate -cell loss in T2D[22,42-44]. Furthermore, an increased number of macrophages are detectable very early in islets, before the onset of diabetes in both high-fat-fed mice and Goto-Kakizaki rats[44]. Although we did not evaluate whether these neuroanatomical and cellular changes in the mouse pancreas from adult offspring are directly associated with a greater predisposition to develop T2D and/or another metabolic disease, the above studies support our hypothesis that these pathological changes may precede the development of metabolic diseases.

Our study demonstrates that an uncontrolled hyperglycemic state during pregnancy induces long-term alterations in pancreatic tissue from adult mouse offspring born from dams with GDM. While the mechanisms behind these GDM-induced long-term complications are unknown, based on the literature we propose these hypotheses. First, the alterations found in the pancreatic tissue from adult offspring could be due to direct toxic effects induced by STZ in the developing embryos since it was injected *i.p.* at the seventh day of gestation. However, cell death in fetuses from dams treated with STZ is similar in magnitude compared to that found in fetuses from dams treated with vehicle[45]. Additionally, while STZ can cross the placenta, it has a very short half-life (7 min). Based on this, it is unlikely that the alterations found result from a direct effect of STZ on the earliest stages of embryonic development[46]. Second, an increased density of sympathetic and sensory nerve fibers could be related to chronic hyperglycemia-induced alterations in the expression/function of neurotrophins and their receptors during fetal growth, which are pivotal for neuronal growth, differentiation, and survival of neurons innervating visceral organs including the pancreas[47,48]. As well as, increased vascularization and macrophage infiltration could result from impaired synthesis of key molecules regulating cell growth, vasculo-

genesis, and angiogenesis. Accordingly, endothelial nitric oxide synthase and *VEGF* genes are increased in embryos from mouse dams with STZ-induced GDM[49].

The present study has some limitations. First, we recognize that while we found cellular alterations in the pancreatic tissue from adult offspring born from dams with GDM, there is a need to assess whether these anatomical changes are translated into functional alterations such as a higher propensity to develop and/or greater severity of metabolic disease. Secondly, it is not possible to discern whether the increase in endomucin$^+$ blood vessels[50] will result in changes in pancreatic blood flow. Third, while we observed an increase in the number of macrophages around acinar cells of offspring of GDM dams; it is unknown whether this macrophage infiltration will lead to altered production of pro-inflammatory and/or anti-inflammatory cytokines. Finally, we do recognize that these anatomical alterations were found at one time-point in life, however, it is warranted to determine when these changes start and/or to assess whether they disappear with aging.

CONCLUSION

We found a significant increase in the density of CGRP$^+$ sensory and TH$^+$ sympathetic axons, as well as a greater vascularization and macrophage infiltration in the exocrine pancreas and an increase in the number of blood vessels in pancreatic islets of female adult offspring born from dams with STZ-induced GDM, but without increased fasting blood glucose levels. Understanding the factors driving this neuroplasticity and cellular/vascular alterations may provide pharmacologic insight and targets for controlling these long-term metabolic complications of GDM in adult progeny.

ARTICLE HIGHLIGHTS

Research background

Epidemiological studies have shown several long-term neurological, cardiovascular, and endocrinological complications of gestational diabetes mellitus (GDM) in offspring.

Research motivation

Although there are several reports about the metabolic long-term complications of GDM on offspring, there is scarce information about the pathological changes at a cellular level that occur in the pancreas of offspring born from dams with GDM.

Research objectives

To quantify a subset of sensory and sympathetic nerve fibers, macrophages, and vasculature in the pancreas from adult offspring born from mouse dams with GDM.

Research methods

GDM was induced by *i.p.* administration of streptozotocin (STZ) in ICR mouse dams. Adult female offspring born from dams with and without GDM were sacrificed and pancreata were processed for immunohistochemistry. There was a quantification of the density of sensory (CGRP) and sympathetic (TH) axons, blood vessels (endomucin), and macrophages (CD68) in the splenic pancreas using confocal microscopy.

Research results

Offspring mice born from STZ-treated dams had similar body weight and blood glucose values compared to offspring born from vehicle-treated dams. However, the density of CGRP$^+$ and TH$^+$ axons, endomucin$^+$ blood vessels, and CD68$^+$ macrophages in the exocrine pancreas was significantly greater in offspring from mothers with GDM *vs* control offspring. Likewise, the microvasculature in the islets was significantly greater, but not the number of macrophages within the islets of offspring born from dams with GDM compared to control mice.

Research conclusions

GDM induces neuronal, vascular, and inflammatory changes in the pancreas of adult progeny, which may partially explain the higher propensity for offspring of mothers with GDM to develop metabolic diseases.

Research perspectives

Future studies are needed to evaluate the functional implications of these alterations and determine if their blockade with mechanism-based therapies may decrease the risk of developing impaired glucose tolerance and type-2 diabetes in individuals born from women with GDM.

FOOTNOTES

Author contributions: Muñoz-Islas E, Jiménez-Andrade JM, and Peters CM designed and coordinated the study; Muñoz-Islas E contributed to funding acquisition; Santiago-SanMartin ED, Mendoza-Sánchez E, Torres-Rodríguez HF, and Ramírez-Quintanilla LY performed the experiments and acquired and analyzed data; Muñoz-Islas E, Jiménez-Andrade JM, and Peters CM drafted the manuscript; and all authors contributed to the interpretation of the results and critical review of the paper, and approved the final version of the article.

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Data sharing statement: Technical appendix, statistical code, and dataset available by request (jandrade@docentes.uat.edu.mx). Not additional data are available.

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ORIGINAL ARTICLE

Basic Study

Icariin accelerates bone regeneration by inducing osteogenesis-angiogenesis coupling in rats with type 1 diabetes mellitus

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Abstract

BACKGROUND

Icariin (ICA), a natural flavonoid compound monomer, has multiple pharmacological activities. However, its effect on bone defect in the context of type 1 diabetes mellitus (T1DM) has not yet been examined.

AIM

To explore the role and potential mechanism of ICA on bone defect in the context of T1DM.

METHODS

The effects of ICA on osteogenesis and angiogenesis were evaluated by alkaline phosphatase staining, alizarin red S staining, quantitative real-time polymerase chain reaction, Western blot, and immunofluorescence. Angiogenesis-related assays were conducted to investigate the relationship between osteogenesis and angiogenesis. A bone defect model was established in T1DM rats. The model rats were then treated with ICA or placebo and micron-scale computed tomography, histomorphometry, histology, and sequential fluorescent labeling were used to evaluate the effect of ICA on bone formation in the defect area.

RESULTS

ICA promoted bone marrow mesenchymal stem cell (BMSC) proliferation and

osteogenic differentiation. The ICA treated-BMSCs showed higher expression levels of osteogenesis-related markers (alkaline phosphatase and osteocalcin) and angiogenesis-related markers (vascular endothelial growth factor A and platelet endothelial cell adhesion molecule 1) compared to the untreated group. ICA was also found to induce osteogenesis-angiogenesis coupling of BMSCs. In the bone defect model T1DM rats, ICA facilitated bone formation and CD31^{hi}EMCN^{hi} type H-positive capillary formation. Lastly, ICA effectively accelerated the rate of bone formation in the defect area.

CONCLUSION

ICA was able to accelerate bone regeneration in a T1DM rat model by inducing osteogenesis-angiogenesis coupling of BMSCs.

Key Words: Icariin; Osteogenesis-angiogenesis coupling; Type 1 diabetes mellitus; Bone defect; Bone regeneration

Core Tip: Type 1 diabetes mellitus (T1DM) leads to a decrease in bone formation in a bone defect area. We demonstrated that icariin, a natural flavonoid compound monomer, accelerated bone regeneration by inducing osteogenesis-angiogenesis coupling of bone marrow mesenchymal stem cells in a T1DM rat model. This finding indicates that further investigations into the effective coupling of osteogenesis and angiogenesis should be undertaken in the field of bone regeneration in T1DM patients.

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INTRODUCTION

Diabetes mellitus (DM) is a growing epidemic globally[1]. Worldwide, about 463 million adults aged 20 years to 79 years are suffering from diabetes, with a prevalence rate of 9.3%. It is estimated that by 2030 there will be 578 million (10.2%) people living with DM[2]. DM places a significant strain on medical resources and patient quality of life, which in turn places heavy burdens on society and the patient's family[3]. DM is typically categorized as type 1 DM (T1DM) and type 2 DM based on the etiology. Patients with T1DM require exogenous insulin to lower blood glucose. Otherwise, chronic high blood glucose can lead to damage in the heart, blood vessels, eyes, kidneys and nerves[4]. A growing body of research has shown T1DM affects bone metabolism[5-7]; however, the underlying mechanism has not yet been fully elucidated.

Although the bone has a propensity for repairing itself, cases arise that are beyond the self-repairing capacity of the bone[8]. One of these cases is a bone defect in a patient with T1DM[9]. Bone metabolism is disordered in T1DM, which increases the challenges for the treatment of bone defects[10-12]. The current standard of treatment are allografts and autografts. However, patients find these therapies to be unsatisfactory[13]. Studies have suggested that the pathogenesis of disordered bone metabolism caused by T1DM is closely related to the imbalance between bone resorption and bone formation, and a high glucose environment could significantly inhibit osteoblast-mediated bone formation and promote osteoclast-mediated bone absorption[14-16].

Icariin (ICA) is a natural flavonol glycoside primarily extracted from *Herba Epimedii*. It has multiple pharmacological activities, including anti-inflammatory, anti-rheumatic, anti-diabetic nephropathy, anti-apoptotic, and anti-oxidative properties[17-21]. ICA can also promote osteogenesis and play an anti-osteoporotic role[22-24]. Huang *et al*[25] showed that ICA promoted osteogenic differentiation through upregulation of BMAL1, and Cheng *et al*[26] demonstrated that ICA attenuated thioacetamide-induced bone loss through downregulation of the RANKL-p38/ERK-NFAT pathway. Hao *et al*[27] observed that ICA accelerated bone regeneration in the defect area of rabbit skulls. Several other studies have demonstrated the protective effects of ICA in T1DM models[28-30]. Considering these findings collectively, we hypothesize that ICA has therapeutic potential for bone defect repair in T1DM.

Both osteogenesis and angiogenesis exert vital roles during the bone regeneration process[28-31]. Several studies have consistently demonstrated the ability of ICA to promote osteogenesis[32-34]. Interestingly, ICA has been shown to play different roles in angiogenesis depending on the circumstances. Yu *et al*[35] reported that ICA promoted angiogenesis in glucocorticoid-induced osteonecrosis of femoral heads, and Huang *et al*[36] demonstated ICA inhibition of angiogenesis *via* regulation of the TDP-43 signaling pathway. It is unknown how ICA affects angiogenesis during the bone regeneration process in the context of T1DM. Therefore, this study investigated the effects and potential mechanisms of ICA on bone defect repair in the context of T1DM.

MATERIALS AND METHODS

Chemicals and reagents

ICA (Cat. No. M211098) was ordered from Mreda Technology Co., Ltd. (Beijing, China). Monoclonal antibodies against CD29 (Cat. No. 11-0291-82), CD90 (Cat. No. 11-0900-81), CD105 (Cat. No. MA1-19594), CD34 (Cat. No. 11-0341-85), and CD45 (Cat. No. 11-0461-82) were purchased from eBioscience (San Diego, CA, United States). Primary antibodies against alkaline phosphatase (ALP; Cat. No. DF6225), osteocalcin (OCN; Cat. No. DF12303), vascular endothelial growth factor A (VEGFA; Cat. No. AF5131), and platelet endothelial cell adhesion molecule 1 (CD31; Cat. No. AF6191) were obtained from Affinity Biosciences (Cincinnati, OH, United States). The primary antibody against endomucin (EMCN; Cat. No. sc-65495) was obtained from Santa Cruz Biotechnology (Dallas, TX, United States).

Cell proliferation assay

Rat bone marrow mesenchymal stem cells (BMSCs) were isolated and cultured as previously described[37]. For phenotypic analysis, expression of CD29, CD90, CD105, CD34, and CD45 was evaluated. Cell counting kit-8 (CCK-8; Cat. No. C0039, Beyotime, Beijing, China) was used to evaluate the effect of ICA on BMSC proliferation. BMSCs were treated with different concentrations of ICA (0 μM, 1 μM, 10 μM, and 100 μM) for 1 d, 3 d, 5 d, and 7 d. The untreated group served as control (CON).

Colony-forming unit assay

BMSCs were seeded into 6-well plates (1×10^3 cells/well) and incubated in the presence or absence of ICA for 1 wk. Then, the clones were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 20 min. Colonies containing 50 or more cells were quantified by ImageJ software (Release 1.51, National Institutes of Health, Bethesda, MD, United States).

Osteogenic differentiation assays

When BMSC confluency reached 80%, the medium was replaced with osteogenic medium. Varying concentrations of ICA were added. Total cellular proteins were extracted, and the supernatant liquid was collected for downstream assays. ALP activity was measured with an ALP Staining Kit (Beyotime) after 1 wk. Calcium mineralization was detected after 3 wk *via* alizarin red S (ARS) solution (Beyotime).

Quantitative real-time polymerase chain reaction

Total RNA was extracted with an RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, United States), and reverse transcription was performed with a cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Green qPCR Master Mix (Thermo Fisher Scientific). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences are listed in Table 1.

Western blot

Total protein was extracted using RIPA lysis buffer with protease inhibitors and protein phosphatase inhibitors (Beyotime) on ice. The cell lysates were collected and ultrasonicated for 10 min. After 15 min of centrifugation (4 °C, 12000 rpm), the protein concentration was measured with a BCA Protein Assay Kit (Beyotime). Equal amounts of protein (30 μg) were subjected to 10% SDS-PAGE and transferred to PVDF membranes (Serva Electrophoresis GmbH, Heidelberg, Germany). The membranes were incubated with primary antibodies against ALP (1:1000 dilution), OCN (1:1000), VEGFA (1:1000), CD31 (1:2000), and β-actin (1:5000). The proteins were visualized by autoradiography and analyzed with ImageJ software.

Immunofluorescence

BMSCs were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.5% Triton X-100 for 15 min, and blocked with 1% bovine serum albumin for 30 min. The cells were incubated with primary antibodies overnight at 4 °C. Subsequently, BMSCs were washed thrice with PBS and incubated with fluorescence-conjugated secondary antibodies for 2 h. Fluorescence images were obtained with a BX63 fluorescence microscope (Olympus, Tokyo, Japan).

Angiogenesis-related assays

To further evaluate the proangiogenesis ability of ICA, BMSCs were treated with 10 μM ICA for 1 wk, and the conditioned medium (CM) was harvested. Human umbilical vein endothelial cells (HUVECs) were also cultured under different conditions: (1) Fresh medium (FM) without ICA (FM + CON group); (2) FM with 1 μM ICA (FM + ICA group); (3) CM without ICA (CM + CON group); and (4) CM with 1 μM ICA (CM + ICA group). HUVECs (Cat. No. iCell-h110) were obtained from iCell Bioscience Inc (Shanghai, China).

The scratch wound assay was conducted with HUVECs seeded into 12-well plates at 2×10^5 cells/well. After confluence, the cells were scratched with a sterile yellow pipette tip and then cultured in the conditions listed above. Images of the wound were taken immediately and 24 h later. Images were analyzed by ImageJ software.

The transwell migration assay was conducted with HUVECs seeded into the upper chamber of 24-well transwell plates (BD Biosciences, Franklin Lakes, NJ, United States) at 3×10^5 cells/well. The culture conditions listed above were added to the lower chamber. After 12 h, the cells that migrated to the lower chamber surface were stained with 0.1% crystal violet for 30 min and measured upon visualization with an inverted microscope (IX73, Olympus).

The tube formation assay was conducted with HUVECs seeded into matrigel precoated 96-well plates at 1.5 × 10^4 cells/well. After 8 h of culture, tube formation was observed *via* an inverted microscope.

Surgery and treatment

Male Wistar rats (280 g ± 15 g; 8-wk-old; Laboratory Animal Center of Southern Medical University, Guangzhou, China) were used in this study. All animal experiments were reviewed and approved by the Animal Ethics Committee of Southern Medical University (Approval No. SMUL2022023, Date of approval: 16 November 2022, Guangzhou, China). All rats were housed in 55% humidity and 22 °C constant temperature under 12-h dark/light cycles. After 1 wk of adaptation, rats in the model group received intraperitoneal injections of streptozotocin (65 mg/kg) as previously described[38] to induce T1DM. Rats in the CON group received vehicle injections.

Blood glucose concentrations were evaluated after 3 d and 7 d. If the blood glucose concentration was higher than 16.7 mmol/L, the rats were diagnosed with T1DM and selected for further studies. Then, the T1DM rats and CON rats were weighed and anesthetized with pentobarbital sodium. The longitudinal approach was used to expose the right proximal tibial metaphysis. A standardized drill hole defect (4 mm diameter and 5 mm deep) was used to create a monocortical defect.

After surgery, according to the random number table method, the T1DM rats were classified into the following two groups: T1DM group (n = 32) and ICA group (n = 32). Rats in the control group were regarded as the CON group (n = 32). The ICA group was treated with ICA (100 mg/kg/d) by gavage for 4 wk, and the rats in the CON group and the T1DM group were treated with equal amounts of normal saline for 4 wk. The therapeutic dose of ICA was determined based on previous experiments where ICA showed protective effects in T1DM rats[39].

Micron-scale computed tomography

Bone repair was evaluated by micron-scale computed tomography (micro-CT; Bruker, Kontich, Belgium). The region of interest was first defined as the bone defect area. After three-dimensional reconstruction, the parameters of bone mineral density, bone volume/tissue volume, trabecular number, and trabecular separation were measured by micro-CT.

Histology staining

After micro-CT imaging, the tibias were decalcified in 10% EDTA for 21 d for subsequent histological analysis. Hematoxylin and eosin staining and Masson's trichrome staining were performed on 5 μm-thick sections. For immunohistochemical staining, 6 μm-thick sections were incubated with primary antibodies against OCN (1:100), VEGFA (1:200), and CD31 (1:200). For immunofluorescence staining, 4 μm-thick sections were incubated with primary antibodies against CD31 (1:500) and EMCN (1:100). Staining was visualized using the BX63 fluorescence microscope.

Sequential fluorescent labeling

All rats were injected subcutaneously with 10 mg/kg calcein (Sigma-Aldrich, St Louis, MO, United States) at 10 d and 3 d before sacrifice[40]. Fluorescent agents were freshly prepared before injection and filtered through a 0.45-μm filter. Tibia samples from each group were collected for hard-tissue slicing and imaged by laser confocal microscopy (FV3000, Olympus).

Statistical analysis

Statistical significance was assessed using two-tailed Student's t-test or analysis of variance. All statistical analyses were performed using SPSS software version 26.0 (IBM Corp., Armonk, NY, United States). Differences were considered statistically significant when the P value was < 0.05. Data were summarized as mean ± SEM.

RESULTS

ICA promoted BMSC proliferation and osteogenic differentiation

Flow cytometry of BMSCs confirmed their identity *via* positive expression for CD29 (99.70%), CD90 (99.51%), and CD105 (99.29%) and negative expression for CD34 (0.94%) and CD45 (0.78%) (Figure 1A). The CCK-8 assay indicated that ICA (Figure 1B) promoted BMSC proliferation at certain concentrations; however, the high concentration (100 μM) showed an inhibitory effect on BMSC proliferation (Figure 1C). The proliferation-promoting concentrations (1 μM and 10 μM) were confirmed by colony forming unit assay (Figure 1D and E). Next, we evaluated the osteogenic ability of ICA *via* ALP and ARS staining. The groups treated with ICA had higher ALP activity, and the optimal concentration was 10 μM (Figure 1F and G). The ICA-treated BMSCs showed more calcium nodule deposits, and the optimal concentration was 10 μM (Figure 1H and I).

ICA enhanced expression of osteogenesis-related and angiogenesis-related markers

After 3 d of incubation in ICA, the gene expression levels of osteogenesis-related markers were evaluated by qRT-PCR. The expression levels of *ALP* and *OCN* were elevated in the ICA-treated groups, but the expression levels of runt-related transcription factor 2 and collagen type I alpha 1 were not significantly different from those in the CON group (Figure 2A). After 1 wk of incubation, the protein levels of ALP and OCN were also elevated in the ICA-treated groups (Figure 2B). Furthermore, immunofluorescence staining of ALP and OCN confirmed these findings (Figure 2C and D). The optimal concentration of ICA was 10 μM, which was consistent with qRT-PCR and western blot. These results

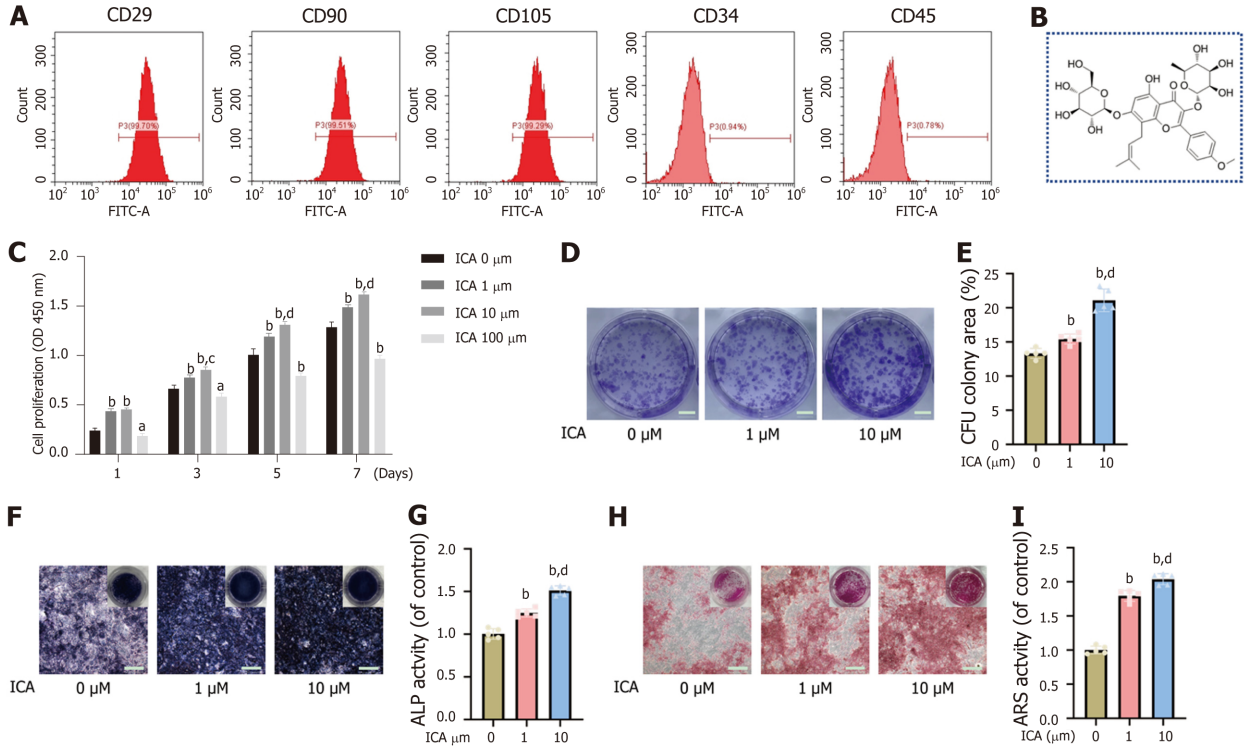

Figure 1 Icariin promoted bone marrow mesenchymal stem cell proliferation and osteogenic differentiation. A: Bone marrow mesenchymal stem cell (BMSC) surface markers were detected by flow cytometry; B: Icariin (ICA) chemical structure; C: The effect of ICA on BMSC proliferation was measured by the cell counting kit-8 assay; D: Representative images of the colony-forming unit (CFU) assay to determine the effect of ICA on BMSC proliferation; E: Quantification of the CFU assay; F: Representative images of alkaline phosphatase (ALP) staining (scale bar: 250 μm); G: ALP activity detection; H: Representative images of alizarin red S (ARS) staining (scale bar: 250 μm); I: Semi-quantitative result of ARS staining. Data are mean ± SEM (n = 5). $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* 1 μM ICA group. ICA: Icariin.

suggested that ICA enhanced the expression of osteogenesis-related markers.

After 3 d of incubation in ICA, the gene expression levels of angiogenesis-related markers were evaluated by qRT-PCR. The expression levels of *VEGFA* and *CD31* were elevated in the ICA-treated groups. However, the expression levels of angiopoietin-2 and angiopoietin-4 detected in the ICA-treated groups were not significantly different from those in the CON group (Figure 3A). After 1 wk of incubation, the protein expression levels of VEGFA and CD31 were also elevated in the ICA-treated groups (Figure 3B). Immunofluorescence staining of VEGFA and CD31 confirmed that ICA enhanced the expression of VEGFA and CD31 (Figure 3C and D). Significantly, the optimal concentration of ICA was also 10 μM. This indicates that the optimal concentration of ICA for promoting osteogenesis and angiogenesis was the same.

ICA induced osteogenesis-angiogenesis coupling of BMSCs

To further evaluate the proangiogenic ability of ICA, HUVECs were assessed *via* angiogenesis-related assays. The results of the scratch wound assay revealed that there was no significant difference in HUVEC migration between the FM + CON group and the FM + ICA group ($P > 0.05$). Interestingly, HUVEC migration was enhanced in the CM + CON group compared to the FM + CON group, as well as in the CM + ICA group compared to the CM + CON group (Figure 4A and B). This migration capacity was confirmed by transwell migration assay (Figure 4C and D). In addition, there were no differences in tube formation in HUVECs of the FM + CON group and FM + ICA group ($P > 0.05$). However, CM increased tube formation, and this effect was even greater in the CM + ICA group compared to the CM + CON group (Figure 4E and F). These findings imply that ICA does not promote angiogenesis in HUVECs in a direct manner but that it can promote angiogenesis in a BMSC-mediated manner. Therefore, the pro-osteogenic effect of ICA is coupled with its proangiogenesis effect.

ICA improved bone repair capacity by promoting osteogenesis in T1DM rats

After the tibial defect operation, the rats received ICA for 4 wk. The effect of ICA on bone defect repair was evaluated after 2 wk and 4 wk of the ICA treatment. The three-dimensional reconstruction revealed that ICA enlarged the area of bone regeneration at both time points (Figure 5A and B). Hematoxylin and eosin staining and Masson's trichrome staining confirmed these results (Figure 5C-F). Accordingly, further quantitative analysis of the defect area revealed that the values of bone mineral density, bone volume/tissue volume, and trabecular number were lowest in the T1DM group, and the value of trabecular separation was highest in the T1DM group (Figure 5G). Thus, while T1DM led to a decrease in bone formation in the tibial defects in this rat model, ICA could improve the bone repair capacity by promoting osteogenesis in the bone defect area.

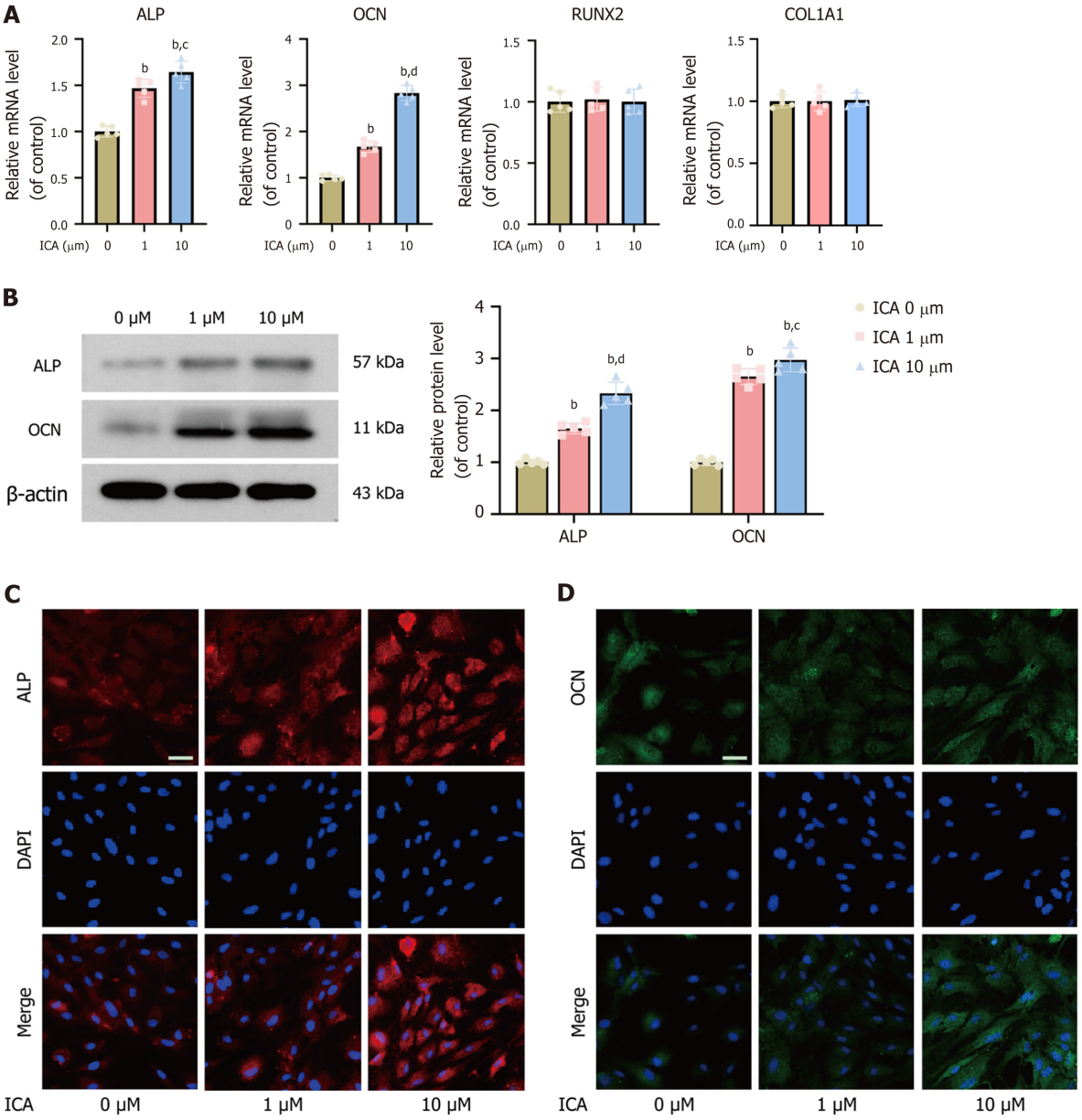

Figure 2 Icariin enhanced the expression of osteogenesis-related markers. A: Expression levels of osteogenesis-related genes [alkaline phosphatase (*ALP*), osteocalcin (*OCN*), runt-related transcription factor 2 (*RUNX2*), and collagen type I alpha 1 (*COL1A1*)] in bone marrow mesenchymal stem cells (BMSCs) following treatment with icariin (ICA); B: Protein expression levels of ALP and OCN by Western blot; C: Expression of ALP by immunofluorescence (scale bar: 100 μm); D: Expression of OCN by immunofluorescence (scale bar: 100 μm). Data are mean ± SEM (n = 5). $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* 1 μM ICA group. ICA: Icariin; ALP: Alkaline phosphatase; OCN: Osteocalcin; RUNX2: Runt-related transcription factor 2; COL1A1: Collagen type I alpha 1; qRT-PCR: Quantitative real-time polymerase chain reaction.

ICA accelerated bone regeneration by inducing osteogenesis-angiogenesis coupling of BMSCs in T1DM rats

At week 2, immunohistochemical staining of the osteogenesis-related marker OCN and the angiogenesis-related markers VEGFA and CD31 revealed an increase in these markers in the ICA group compared to those in the T1DM group (Figure 6A-C). At week 4, the same trend was observed for these markers (Figure 6D-F). The CD31 and EMCN double immunofluorescence staining revealed that ICA facilitated CD31^{hi}EMCN^{hi} type H-positive capillary formation in the bone defect area at both time points (Figure 6G and H). Furthermore, sequential fluorescent labeling revealed that the distance between double labels was wider in the ICA group than in the T1DM group (Figure 6I), which indicated that ICA accelerated the rate of bone formation in the defect area. Quantitative analysis of the bone mineral apposition rate confirmed the above finding (Figure 6J), indicating that ICA accelerated bone regeneration by inducing the osteogenesis-angiogenesis coupling in the T1DM rat model.

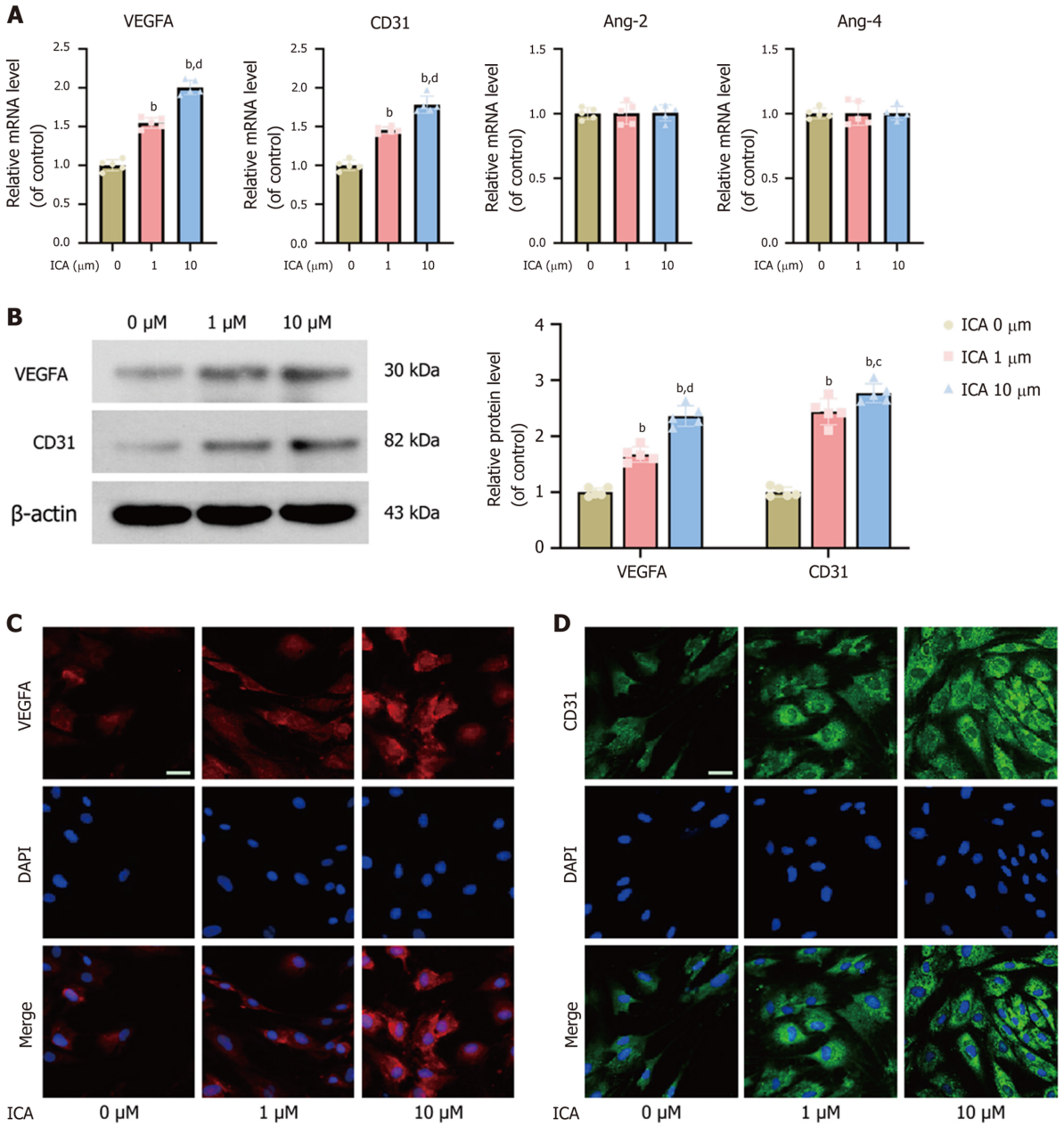

Figure 3 Icariin enhanced the expression of angiogenesis-related markers. A: Expression levels of angiogenesis-related genes [vascular endothelial growth factor A (*VEGFA*), platelet endothelial cell adhesion molecule 1 (*CD31*), angiopoietin-2 *(Ang-2*), and angiopoietin-4 *(Ang-4*)] in bone marrow mesenchymal stem cells (BMSCs) following treatment with icariin (ICA); B: Protein expression levels of VEGFA and CD31 by Western blot; C: Expression of VEGFA by immunofluorescence (scale bar: 100 μm); D: Expression of CD31 by immunofluorescence (scale bar: 100 μm). Data are mean ± SEM (n = 5). $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* 1 μM ICA group. ICA: Icariin; VEGFA: Vascular endothelial growth factor A; CD31: Platelet endothelial cell adhesion molecule 1; Ang-2: Angiopoietin-2; Ang-4: Angiopoietin-4; qRT-PCR: Quantitative real-time polymerase chain reaction.

DISCUSSION

It was widely accepted that the relationship between osteogenesis and angiogenesis is unidirectional[41]. However, later studies indicated that the relationship is actually closely coordinated[42-44]. It is worth noting that DM normally impairs angiogenesis[45-47]. Chinipardaz *et al*[48] observed that angiogenesis was significantly reduced in a fractured region in a T1DM mouse model compared to that in normal mice. The decrease in angiogenesis could suppress trabecular bone regeneration and delay bone healing[49]. Therefore, it is essential to focus on vascular regeneration when studying bone regeneration. Through this study, we discovered that ICA enhanced the expression of osteogenesis-related and angiogenesis-related markers in a T1DM mouse model, and the optimal concentration for promoting osteogenesis and angiogenesis was the same. In addition, although ICA cannot directly promote angiogenesis our results indicated that it can work synergistically with BMSCs to promote angiogenesis.

The connection between BMSCs and endothelial cells is attributed to osteoblastic and angiogenic factor production (*e.g.*, VEGFA)[50], which was consistent with our *in vitro* findings. Our subsequent *in vivo* studies revealed that ICA promoted osteogenesis and H-positive capillary formation in the defect area. Previous studies have observed that H-type

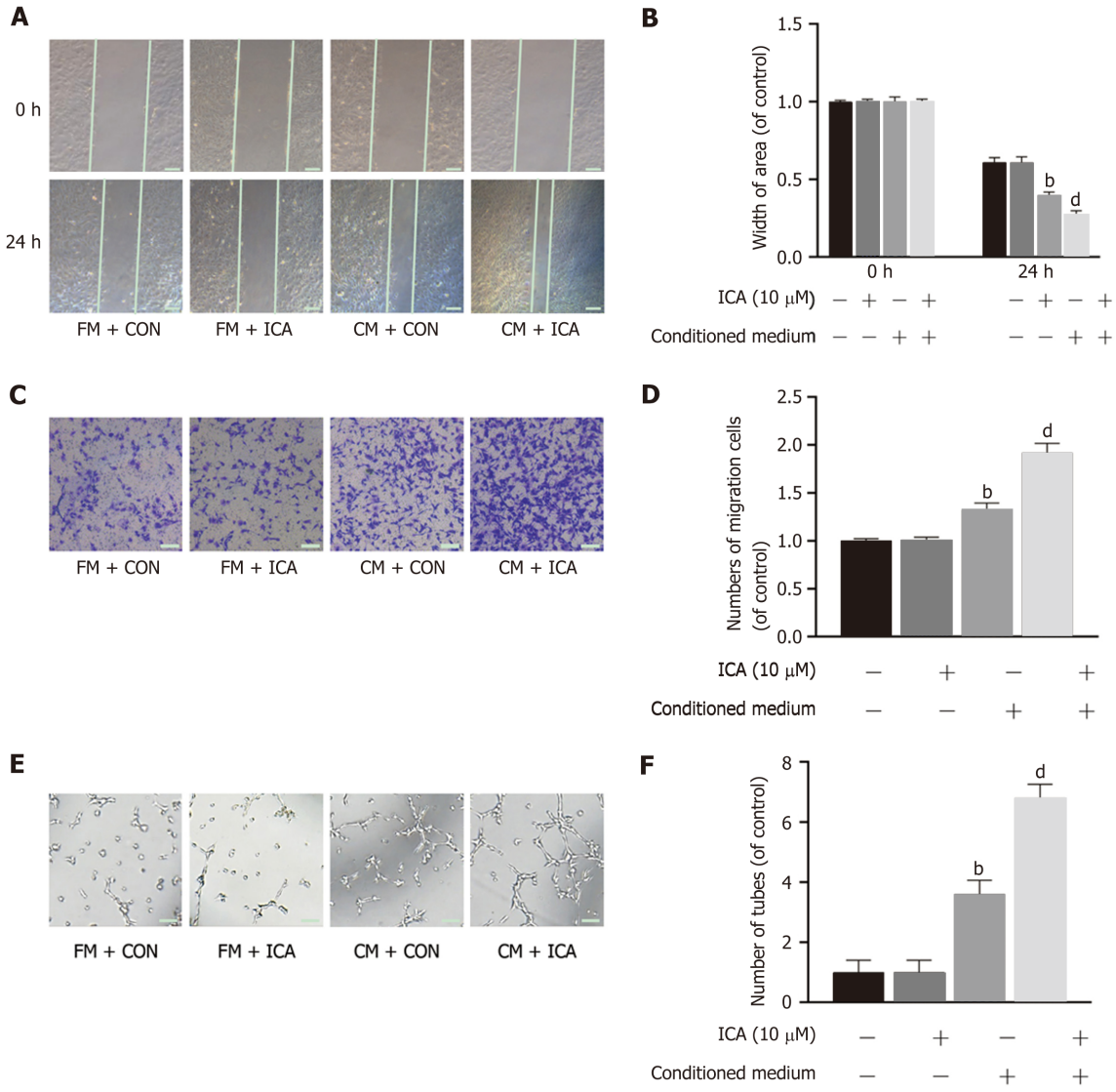

Figure 4 Icariin induced osteogenesis-angiogenesis coupling of bone marrow mesenchymal stem cells. A: Scratch wound assay to show migration of human umbilical vein endothelial cells (HUVECs) cultured with fresh medium (FM) or conditioned medium (CM) and with or without icariin (ICA) (scale bar: 100 μm); B: Quantification of the scratch wound assays; C: Transwell migration assay of HUVECs cultured with FM or CM and with or without ICA (scale bar: 100 μm); D: Quantification of the transwell migration assays; E: Tube formation assay of HUVECs cultured with FM or CM and with or without ICA (scale bar: 100 μm); F: Quantification of the tube formation assays. Data are mean ± SEM (n = 5). $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* CM + control group. ICA: Icariin; FM: Fresh medium; CM: Conditioned medium; Con: Control.

blood vessels could couple osteogenesis and angiogenesis[51-53]. This indicated that the roles of ICA in promoting osteogenesis and angiogenesis were not independent *in vivo*. They are coupled by H-type blood vessels to play synergistic roles. This study is the first to demonstrate that ICA possesses the ability to induce osteogenesis-angiogenesis coupling in BMSCs.

Natural products with structural diversity and biological activity are important sources of innovative drugs[54]. ICA is a natural flavonol glycoside used in traditional Chinese medicine[55] and research has shown that it can promote osteogenesis in various ways[56-58]. Xia *et al*[59] found that ICA could promote osteogenic differentiation of BMSCs by upregulating GLI-1, and Luo *et al*[60] observed that ICA restored osteogenic differentiation of BMSCs in ovariectomized (commonly known as OVX) rats. A growing body of research has shown that ICA is beneficial in models of diabetes[61-63]. Significantly, the safety of ICA has been demonstrated by multiple studies[64-66]. Thus, ICA is expected to exert more vital roles in improving diabetes and promoting bone regeneration due to its natural origin and safety.

The osteogenic differentiation of BMSCs is important for bone regeneration, and angiogenesis plays an indispensable role during the bone regeneration process[67-69]. Wu *et al*[70] showed that ICA could promote repair of a normal bone defect *via* enhancement of osteogenesis and angiogenesis. In this study, we demonstrated that ICA induced osteogenesis-angiogenesis coupling in BMSCs *in vitro*, and that ICA facilitated bone formation and CD31^{hi}EMCN^{hi} type H-positive capillary formation in the defect area of a T1DM rat model. We used single and double fluorochrome labeling as a direct histologic marker of bone formation. This test showed that in the T1DM model there was a reduced rate of bone formation in the defect area compared to the CON group. When the T1DM rats were treated with ICA, the double fluorochrome labeling demonstrated that ICA accelerated the rate of bone formation in the defect area. Although this study

Table 1 Real-time polymerase chain reaction primer sequences

Gene	Forward primer	Reverse primer
ALP	ACCATTCCCACGTCTTCACATTT	AGACATTCTCTCGTTCACCGCC
OCN	GTCAGACTACAACATCCAGAAG	CGAGTATCTTCCTGTTTGACC
RUNX2	GAGCGTTCAACGGCACAG	GACAGTAGACTCCACGACA
COL1A1	TGTCGTTCAACGGCACAG	TGTGGTAGACTCCACGACA
VEGFA	TCAGGAGGACCTTGTGTGATCAG	CATTGCTCTGTACCTTGGGAA
CD31	CACCGTGATACTGAACAGCAA	GTCACAATCCCACCTTCTGTC
Ang-2	GAAGAAGGAGATGGTGGAGA	CGTCTGGTTGAGCAAACTG
Ang-4	GCTCCTCAGGGCACCAAGTTC	CACAGGCGTCAAACCACCAC
GAPDH	GGCATGGACTGTGGTCATGAG	TGCACCACCAACTGTTAGC

ALP: Alkaline phosphatase; *Ang-2*: Angiopoietin-2; *Ang-4*: Angiopoietin-4; *CD31*: Platelet endothelial cell adhesion molecule 1; *COL1A1*: Collagen type I alpha 1; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; *OCN*: Osteocalcin; *RUNX2*: Runt-related transcription factor 2; *VEGFA*: Vascular endothelial growth factor A.

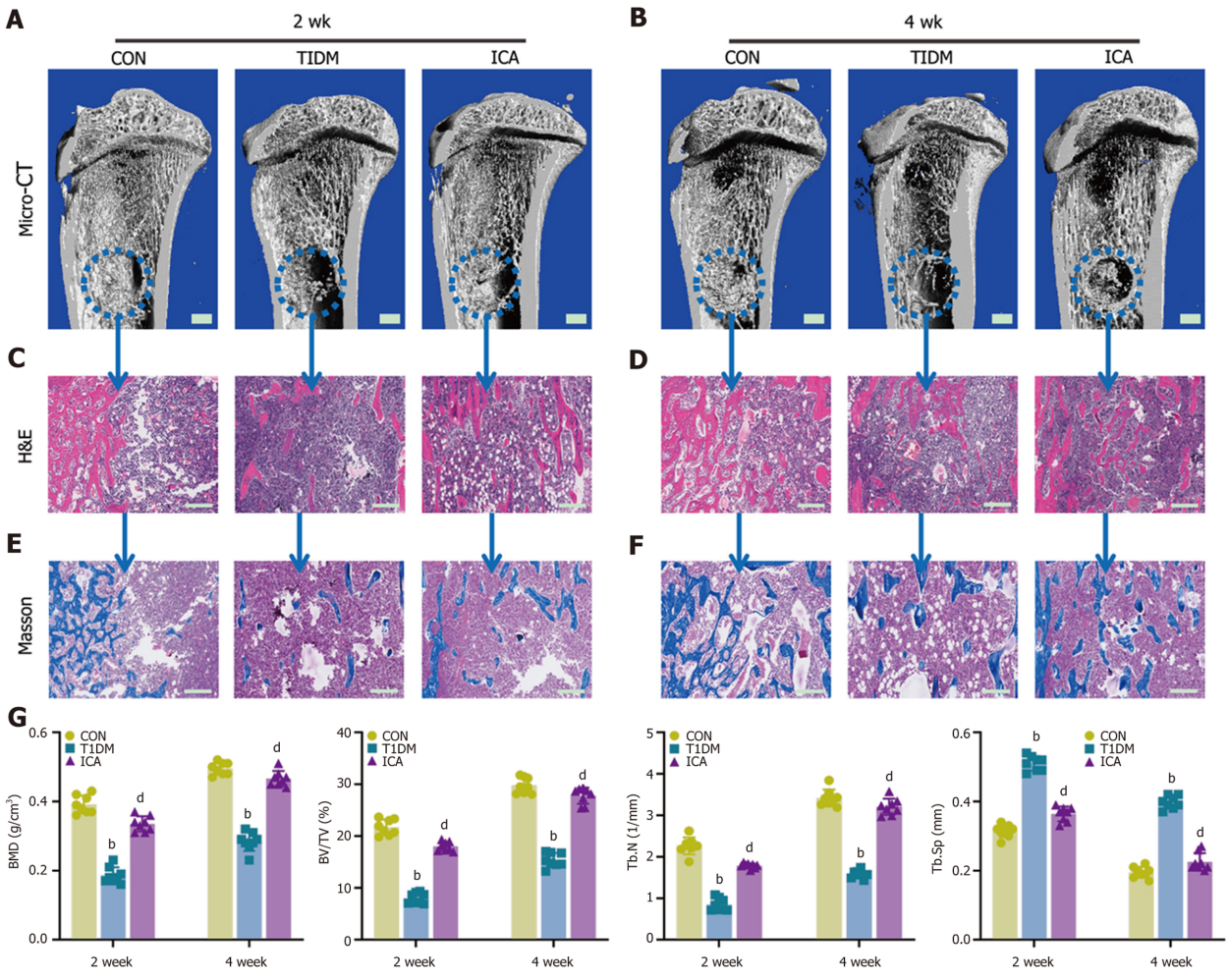

Figure 5 Icariin improved bone repair capacity by promoting osteogenesis in type 1 diabetes mellitus rats. A and B: Three-dimensional reconstruction images of the defect area at week 2 (A) and week 4 (B) (scale bars: 1 mm); C and D: Hematoxylin and eosin staining of the defect area at week 2 (C) and week 4 (B) (scale bars: 200 μm); E and F: Masson's trichrome staining of the defect area at week 2 (E) and week 4 (F) (scale bars: 200 μm); G: Micron-scale computed tomography analysis of bone mineral density (BMD), bone volume/tissue volume (BV/TV), trabecular number (Tb.N), and trabecular separation (Tb.Sp) at weeks 2 and 4. Data are mean ± SEM of the mean (n = 8). $^{b}P < 0.01$ *vs* the control group; $^{d}P < 0.01$ *vs* the type 1 diabetes mellitus group. ICA: Icariin; Con: Control; T1DM: Type 1 diabetes mellitus.

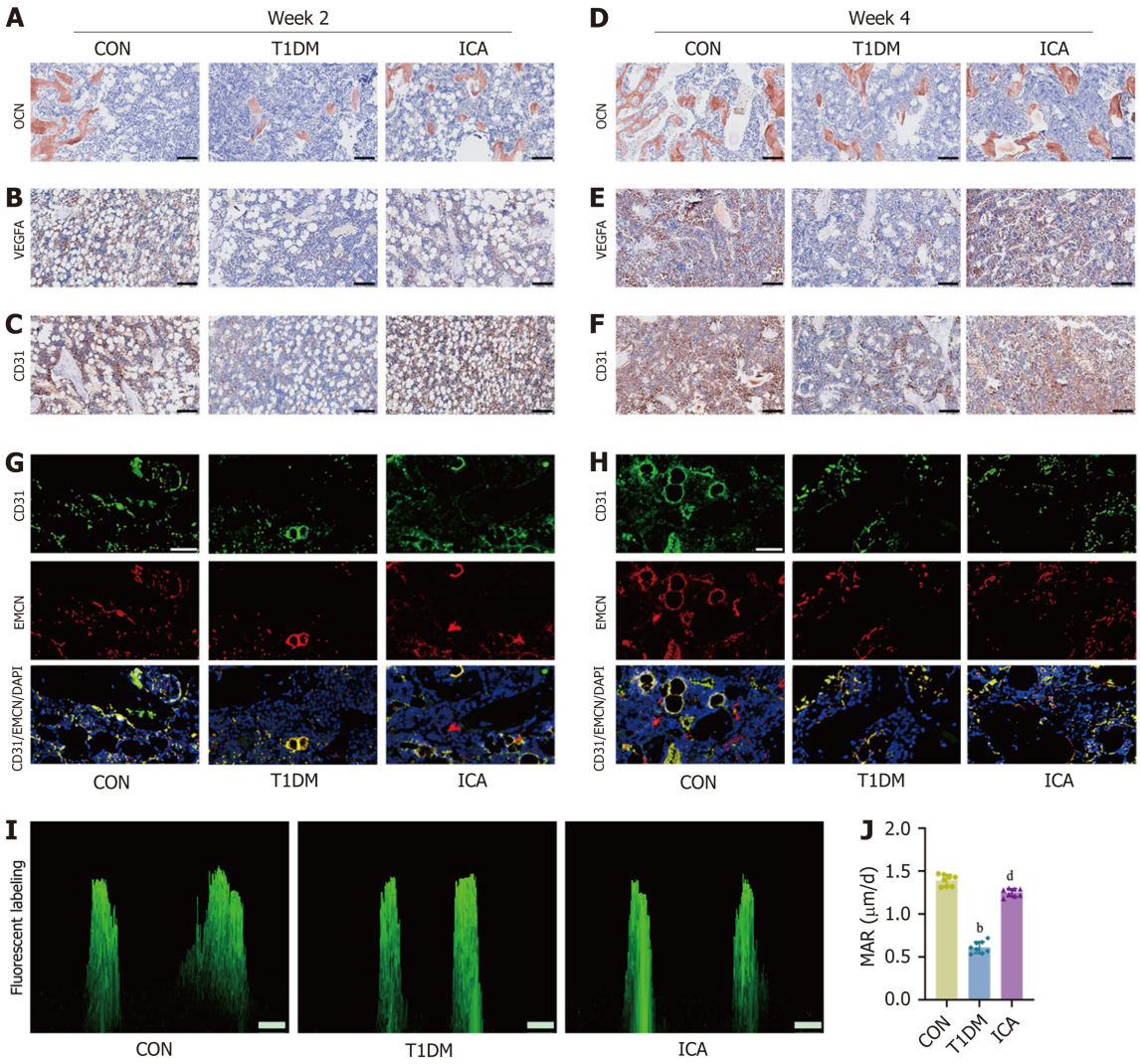

Figure 6 Icariin accelerated bone regeneration by inducing osteogenesis-angiogenesis coupling of bone marrow mesenchymal stem cells in type 1 diabetes mellitus rats. A-C: Immunohistochemical results of osteocalcin (OCN) (A), vascular endothelial growth factor A (VEGFA) (B), and platelet endothelial cell adhesion molecule 1 (CD31) (C) in the defect area at week 2 (scale bars: 100 μm); D-F: Immunohistochemical results of OCN (D), VEGFA (E), and CD31 (F) in the defect area at week 4 (scale bars: 100 μm); G: CD31 and endomucin (EMCN) double immunofluorescence staining in the defect area at week 2 (scale bar: 100 μm); H: CD31 and EMCN double immunofluorescence staining in the defect area at week 4 (scale bar: 100 μm); I: Sequential fluorescent labeling (scale bar: 10 μm); J: Quantitative analysis of mineral apposition rate. Data are mean ± SEM (n = 8). $^{b}P < 0.01$ *vs* the control group; $^{d}P < 0.01$ *vs* the type 1 diabetes mellitus group. ICA: Icariin; Con: Control; T1DM: Type 1 diabetes mellitus; OCN: Osteocalcin; VEGFA: Vascular endothelial growth factor A; CD31: Platelet endothelial cell adhesion molecule 1.

revealed that the pro-osteogenesis effect of ICA is strictly connected to its pro-angiogenesis effect and together contribute to the bone regeneration in the context of TIDM, the effects of ICA on bone resorption and bone homeostasis were not investigated; indeed, these latter topics are the focus of our ongoing research.

CONCLUSION

Taken together, we conclude that ICA could accelerate bone regeneration by inducing osteogenesis-angiogenesis coupling of BMSCs in the T1DM rat model. This finding also implies that ICA may be a potential drug for treating bone defect in the context of TIDM, and more importantly that further investigations into the effective coupling of osteogenesis and angiogenesis should be undertaken in the field of bone regeneration in T1DM patients.

ARTICLE HIGHLIGHTS

Research background

Icariin (ICA) has multiple pharmacological activities. However, its effect on bone defect repair in the context of type 1 diabetes mellitus (T1DM) remains unclear.

Research motivation

ICA possesses the ability to promote osteogenesis and exert protective effects in T1DM. Therefore, ICA may have therapeutic potential for repairing bone defects in patients with T1DM.

Research objectives

To explore the role of ICA on bone defect repair in T1DM models.

Research methods

The effects of ICA on osteogenesis and angiogenesis were evaluated by molecular biology techniques *in vitro*. After a T1DM rat model was established, we evaluated the effect of ICA on bone formation in a defect area.

Research results

ICA promoted bone marrow mesenchymal stem cell (BMSC) osteogenic differentiation and induced osteogenesis-angiogenesis coupling of BMSCs *in vitro*. Subsequently, we observed that ICA facilitated bone formation and type H vessel formation in the defect area of the T1DM rat model. Sequential fluorescent labeling confirmed that ICA could effectively accelerate the rate of bone formation in the defect area.

Research conclusions

ICA accelerated bone regeneration by inducing osteogenesis-angiogenesis coupling of BMSCs in the T1DM rat model.

Research perspectives

Our study highlighted the importance of effective coupling of osteogenesis and angiogenesis in bone regeneration in the context of T1DM.

FOOTNOTES

Co-corresponding authors: Jia Zheng and Yi-Kai Li.

Author contributions: Zheng J and Li YK contributed equally to this work and are co-corresponding authors on this paper according to the critical roles they played throughout the research in experimental design, data analysis, and provision of intellectual ideas and methods; Zheng S, Zheng J, and Li YK designed the research study; Zheng S, Hu GY, and Li JH performed the research; Li JH and Zheng J contributed new reagents and analytic tools; Zheng S, Zheng J, and Li YK analyzed the data and wrote the manuscript; and all authors read and approved the final manuscript.

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Data sharing statement: Data will be made available upon reasonable request to the corresponding author.

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

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META-ANALYSIS

Application of three-dimensional speckle tracking technique in measuring left ventricular myocardial function in patients with diabetes

Zheng Li, Ying Qian, Chun-Yun Fan, Yong Huang

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Abstract

BACKGROUND

Diabetic cardiomyopathy is considered as a chronic complication of diabetes mellitus (DM). Therefore, early detection of left ventricular systolic function (LVSF) damage in DM is essential.

AIM

To explore the use of the three-dimensional speckle tracking technique (3D-STI) for measuring LVSF in DM patients *via* meta-analysis.

METHODS

The electronic databases were retrieved from the initial accessible time to 29 April 2023. The current study involved 9 studies, including 970 subjects. We carried out this meta-analysis to estimate myocardial function in DM compared with controls according to myocardial strain attained by 3D-STI.

RESULTS

Night articles including 970 subjects were included. No significant difference was detected in the left ventricular ejection fraction between the control and the diabetic group ($P > 0.05$), while differences in global longitudinal strain, global circumferential strain, global radial strain, and global area strain were markedly different between the controls and DM patients (all $P < 0.05$).

CONCLUSION

The 3D-STI could be applied to accurately measure early LVSF damage in patients with DM.

Key Words: Diabetes mellitus; Left ventricular systolic dysfunction; Three-dimensional speckle tracking echocardiography; Meta analysis

Core Tip: In this study, we found that three-dimensional speckle tracking technique (3D-STI) could precisely assess early left ventricular systolic dysfunction in diabetes mellitus (DM). Our meta-analysis indicated that global longitudinal strain (GLS), global radial strain, global circumferential strain, and global area strain (GAS) in DMs were lower than controls, suggesting that the left ventricular systolic function in DMs was impaired compared with controls. Among them, the decrease of GLS and GAS was more obvious, which may be since the left ventricular wall is composed of three layers of myocardial fibers. The assessment of left ventricular strain in DM patients through 3D-STI might estimate the damage of left ventricular systolic function in DM in the early stage.

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INTRODUCTION

Diabetes mellitus (DM) is a common disease in China. Long-term poor blood glucose control can cause multisystem damage and a series of chronic complications[1]. Diabetic cardiomyopathy (DCM) is a chronic complication and is a serious cause of poor prognosis in individuals with DM[2]. Additionally, numerous reports have suggested that DM could elevate the occurrence of cardiac disorders, hypertension, and other illnesses and could worsen coronary artery illness[3]. Therefore, early detection of left ventricular systolic function (LVSF) damage in DM patients is essential.

Currently, the routine clinical factor for assessing cardiac function is left ventricular ejection fraction (LVEF). However, LVEF is strongly affected by subjectivity, and several publications have exposed that LVEF could not indicate the severity of LVSF in patients with earlier DM. Moreover, it is impossible to effectively predict patients with segmental wall motion abnormalities and ejection fraction retention by LVEF. Therefore, evaluating left ventricular myocardial function is highly important for the diagnosis, treatment, and prognosis of heart disease. The 3D-STI is an innovative approach for evaluating cardiac motor function. It tracks myocardial motion from three-dimensional space through detecting myocardial echo speckle signals, which breaks through the limitation of the two-dimensional plane of 2D-STI and can evaluate cardiac function more accurately.

The 3D-STI is of great value in the assessment of primary or secondary LVSF. However, the ability of the 3D-STI to evaluate the outcome of left ventricular myocardial contractile function (LVMCF) in DM remains uncertain, and additional reports are needed. The purpose of the current analysis was to examine the ability of the 3D-STI to early predict LVSF damage in DM *via* meta-analysis.

MATERIALS AND METHODS

Screening of articles

The meta-analysis was registered (202390079) in INPLASY and followed the preferred reporting criteria of PRISMA 2020. A comprehensive exploration of studies on 3D-STI assessment in DM patients was conducted based on the PRISMA 2020 recommendations. Through the PubMed, Embase, Scopus, and Cochrane Library databases, studies on 3D-STI assessment in DM patients were retrieved from the initial obtainable time to 29 April 2023. The exploration strategy was employed based on the following terms: (1) "Three-dimensional speckle tracking", "3D-speckle tracking", "3D-STI", or "STE"; (2) "Diabetes mellitus" or "DM"; and (3) "left ventricular" or "LV". The current study conducted a meta-analysis.

Data extraction and quality evaluation

Full-text articles that included the main factors were eligible for inclusion in this study. The main factors were as follows: (1) Had a randomized controlled trial and cohort study; (2) had an article comparing LVMCF parameters between the DM group and control group; (3) had no history of cardiovascular syndromes; (4) had a diagnostic approach of 3D-STI; and (5) had at least one notable result, including global longitudinal strain (GLS), global circumferential strain (GCS), global radial strain (GRS), and global area strain (GAS).

Repeated documents and publications that did not supply original descriptions of interest, such as case reports, meeting essays, reviews, fundamental studies, and nonrelevant publications, were eliminated. Two researchers individually assessed the selected articles followed the selection criteria. Divergences between researchers were resolved

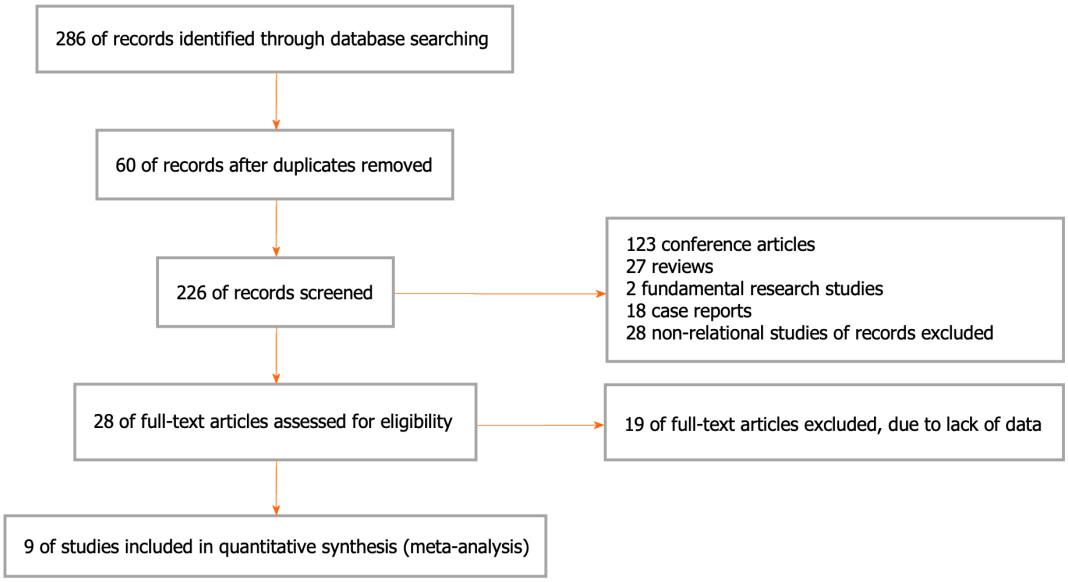

Figure 1 Study selection procedure.

by an agreement obtained from the assist of a third author.

The quality of the publications was considered with the New-Ottawa Scale and evaluated based on the following features: The comparison of the case and control groups, and the estimation of the consequences. The quality of the chosen studies was estimated individually by two investigators, and the incongruity was determined by discussion.

Risk of bias evaluation and sensitivity analysis

Publication bias was estimated through Egger's test for the included articles. The random effects approach was used to minimize the variability among the included publications. The stability of the strains was measured through sensitivity analysis by eliminating one by one from the article.

Statistical analysis

The weighted mean difference and 95% confidence interval (95%CI) were employed to depict the statistical consequences of continuous variables. Heterogeneity was measured through RevMan 5.3 software and the I^2 test. An I^2 < 25% indicated low heterogeneity, while a value > 50% implied high heterogeneity. The random-effects model was employed and checked with the fixed-effects model. Sensitivity analyses were carried out through the leave-one-out method. A difference was statistically significant at $P < 0.05$.

RESULTS

Study searching and selection

Two hundred and eighty-six articles were obtained from the above databases using a retrieval strategy. Sixty duplicated studies were excluded. Moreover, studies without suitable information, including meeting articles (123), reviews (27), fundamental studies (2), case reports (18), and nonrelational studies (28), were disqualified. After the full texts were read, 19 studies were rejected due to lack of statistics. Ultimately, the remaining 9 articles were involved. The article collection process is exhibited in Figure 1 and Table 1.

Comparison of the LVMCF based on LVEF

In total, 7 articles compared the LVEF measured by 3D-STI between the DMs and controls. The findings showed that the difference in LVEF between DMs and healthy controls was not statistically significant (MD: -1.85, 95%CI: -2.48 to -1.22, $P = 0.46$; $I^2 = 0\%$; Figure 2).

Comparison of the LVMCF based on GLS

Furthermore, all 9 included studies reported GLS in DM patients and healthy controls. The results demonstrated that the LVGLS (MD: 1.41, 95%CI: 1.11 to 1.71, $P = 0.000$; $I^2 = 94\%$; Figure 3) was appreciably lower in the DMs than in the controls.

Comparison of LVMCF based on GCS

There were 9 articles recording the GCS score in patients with DM and controls. The results demonstrated that the LVGCS (MD: 0.02, 95%CI: -0.36 to 0.39, $P = 0.000$; $I^2 = 92\%$; Figure 4) was markedly lower in the DMs than in the controls.

Table 1 General data and quality evaluation of the included studies

Ref.	Country	Instrument	Groups	Number	Gender (male/female, *n*)	Age (yr)	3D-STI parameters and LVEF	NOS score
Tadic *et al*[4], 2015	Serbia	GE Vivid E7	DM	50	26/24	52.00 ± 8.00	GLS, GCS, GRS, GAS, LVEF	8
			NC	50	24/26	50.00 ± 7.00		
Wang *et al*[5], 2015	China	GE Vivid E9	DM	46	24/22	63.10 ± 9.80	GLS, GCS, GRS, GAS, LVEF	7
			NC	40	21/19	65.50 ± 5.90		
Zhang *et al*[6], 2013	China	GE Vivid E9	DM-a[1]	31	15/16	61.00 ± 9.00	GLS, GCS, GRS, GAS, LVEF	8
			DM-b[1]	37	21/16	60.00 ± 10.00		
			NC	63	30/33	58.00 ± 10.00		
Enomoto *et al*[7], 2016	Japan	Aplio-ArtidaTM	DM	77	53/24	56.00 ± 15.00	GLS, GRS, GCS, LVEF	7
			NC	35	18/17	52.00 ± 16.00		
Wang *et al*[8], 2015	China	GE Vivid E9	DM-a[2]	36	18/18	64.40 ± 7.90	GLS, GRS, GCS, GAS	8
			DM-b[2]	41	21/20	65.70 ± 9.00		
			DM-c[2]	46	22/24	63.10 ± 9.80		
			NC	36	18/18	66.80 ± 8.40		
Yang *et al*[9], 2021	China	GE Vivid E9	DM-a[3]	28	19/9	51.42 ± 8.94	GLS, GRS, GCS, LVEF	8
			DM-b[3]	19	13/6	52.16 ± 9.22		
			NC	27	18/9	49.93 ± 8.28		
Conte *et al*[10], 2013	Italy	GE Vivid E7	DM-a[4]	44	23/21	60.90 ± 6.60	GLS, GRS, GCS, GAS	7
			DM-b[4]	27	17/10	56.20 ± 7.80		
			NC	24	13/11	58.40 ± 9.40		
Abomandour *et al*[11], 2022	Egypt	Vivid E2013	DM-a[5]	31	17/14	32.94 ± 5.56	GLS, GRS, GCS, LVEF	7
			DM-b[5]	31	18/13	28.74 ± 9.35		
			NC	31	11/20	30.32 ± 9.53		
Wang *et al*[12], 2022	China	GE Vivid E9	DM-a[6]	40	21/19	64.40 ± 7.90	GLS, GRS, GCS, GAS, LVEF	8
			DM-b[6]	40	21/19	60.80 ± 8.10		
			NC	40	20/20	61.90 ± 6.90		

[1]Diabetes was divided into two subcategories based on a hemoglobin A1c (HbA1c) < 7.0% and an HbA1c ≥ 7.0%.

[2]Diabetes was divided into two subcategories based on an HbA1c < 6.5% and an HbA1C ≥ 6.5%.
[3]Diabetes was divided into two subcategories based on the presence or absence of microvascular complications.
[4]Diabetes status was divided into two subcategories based on body mass index (BMI) < 30 kg/m^2 and BMI ≥ 30 kg/m^2.
[5]Diabetes status was divided into two subcategories: Obese and nonobese.
[6]Diabetes was divided into two subcategories based on the presence or absence of nonalcoholic fatty liver.
DM: Diabetes mellitus; 3D-STI: Three-dimensional speckle tracking technique; GLS: Global longitudinal strain; LEVF: Left ventricular ejection fraction; GCS: Global area strain; GAS: Global area strain; GRS: Global radial strain; NOS: New-Ottawa Scale.

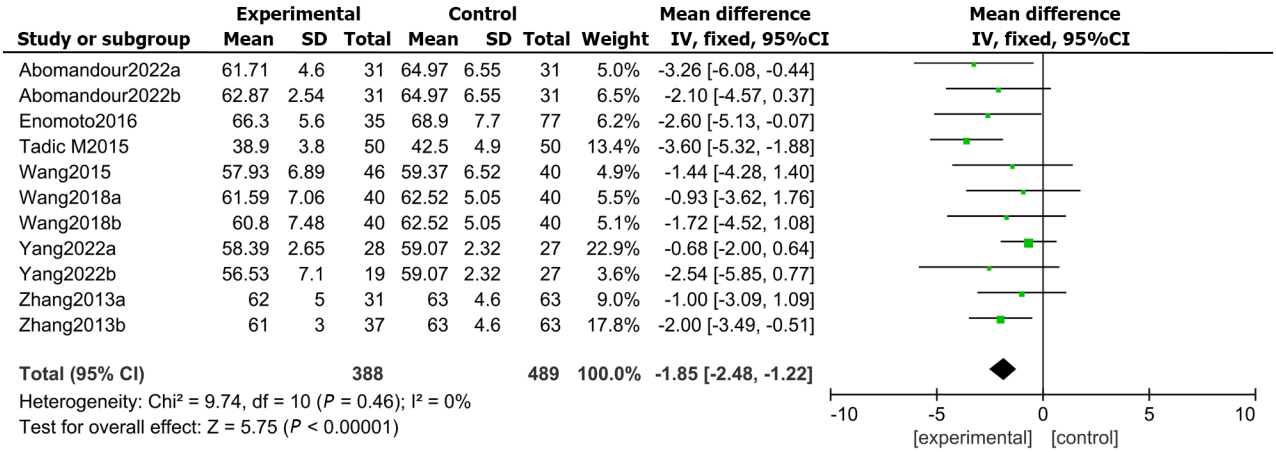

Figure 2 Forest plot showing the comparison of the left ventricular myocardial contractile function between diabetes mellitus and controls based on left ventricular ejection fraction. 95%CI: 95% confidence interval.

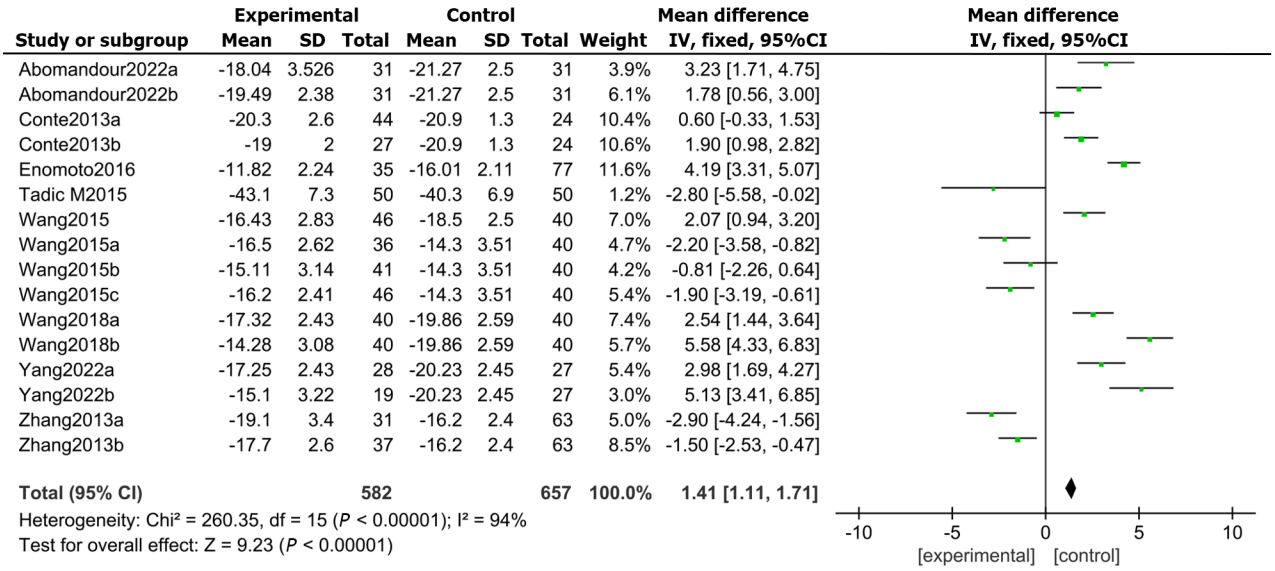

Figure 3 Forest plot showing the comparison of the left ventricular myocardial contractile function between diabetes mellitus and controls based on global longitudinal strain. 95%CI: 95% confidence interval.

Comparison of LVMCF based on GRS

All 9 included studies reported GRSs in DM patients and healthy controls. The results demonstrated that the LVGRS (MD: -1.61, 95%CI: -2.33 to -0.89, P = 0.000; I^2 = 88%; Figure 5) was markedly impaired in the DMs compared with the controls.

Comparison of LVMCF based on GAS

In total, 6 articles compared the GASs measured by 3D-STI between the DM and the control group. The results exposed that GAS was markedly impaired in DMs compared with controls (MD: 0.14, 95%CI: -0.33 to 0.61, P = 0.000; I^2 = 90%; Figure 6).

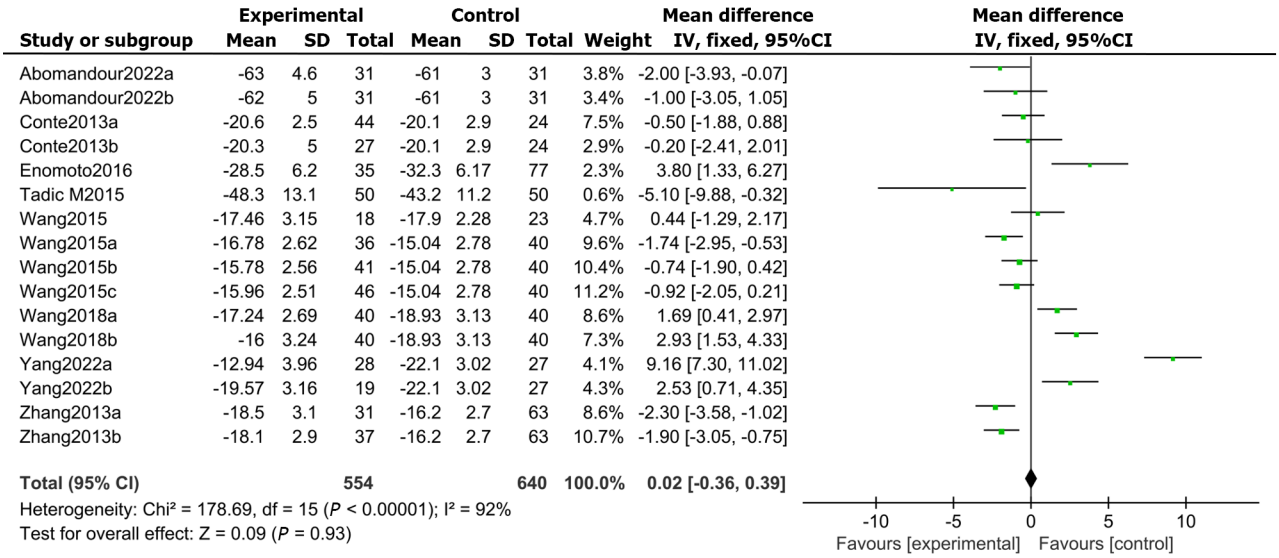

Figure 4 Forest plot comparing the left ventricular myocardial contractile function between diabetes mellitus and controls based on the global circumferential strain score. 95%CI: 95% confidence interval.

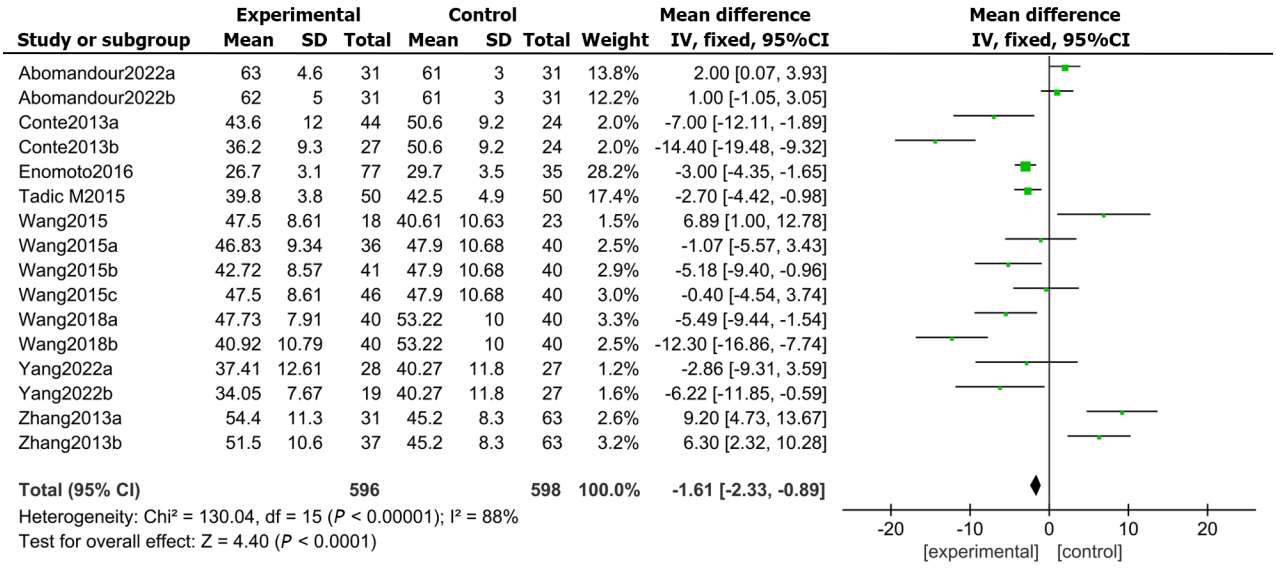

Figure 5 Forest plot showing the comparison of left ventricular myocardial contractile function between diabetes mellitus and controls based on global radial strain. 95%CI: 95% confidence interval.

Risk of bias assessment and sensitivity analysis

Publication bias was evaluated through Egger's test for the involved studies (Figure 7). We detected no publication bias in GLS (P = 0.286) or LVEF (P = 0.825). Moreover, there was publication bias for the GRS (P = 0.022), GCS (P = 0.032), and GAS (P = 0.041). The trim-and-fill approach was further employed to find the modified merged values for the GRS, GCS, and GAS. A sensitivity analysis was also carried out to evaluate the stability of the strains. None of the articles confirmed a marked influence on the merged value, implying that the involved publications exhibited worthy stability.

DISCUSSION

In the early stage of DCM, due to abnormal metabolism of subendothelial cardiomyocytes and more severe hypoxia, myocardial fibers are first affected[13]. When damaged, the strain parameter GLS reflects myocardial mechanics abnormalities[14]. However, due to the inconsistency of the duration of disease and the degree of blood glucose control in different patients, GLS and other left ventricular systolic strain parameters (GCS, GRS, and GAS) can decrease at the same time or successively[15]. Therefore, the application of appropriate and accurate diagnostic tools to detect and monitor myocardial injury is an important aspect of managing DCM patients.

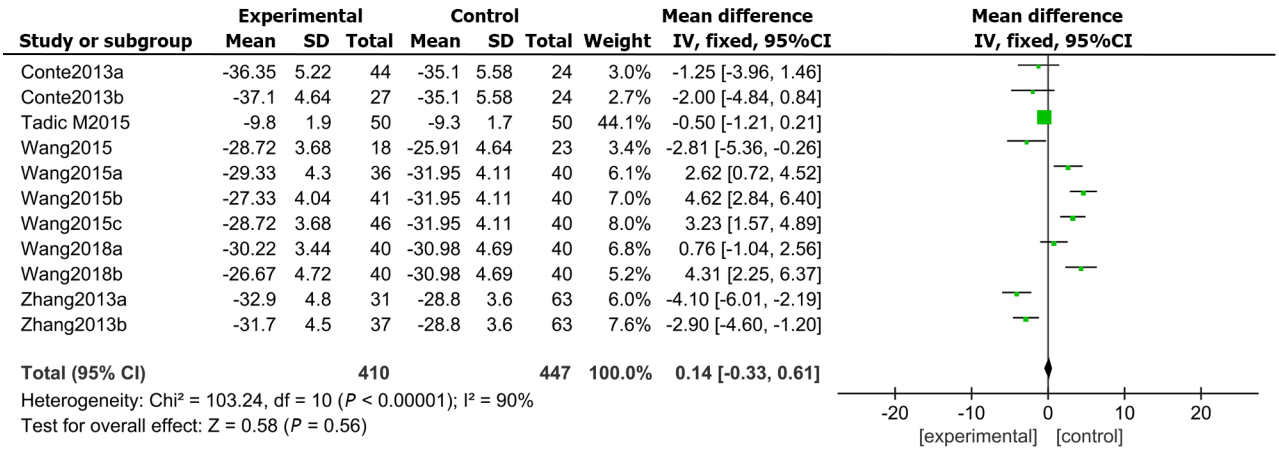

Figure 6 Forest plot showing the comparison of left ventricular myocardial contractile function between diabetes mellitus and controls based on global area strain. 95%CI: 95% confidence interval.

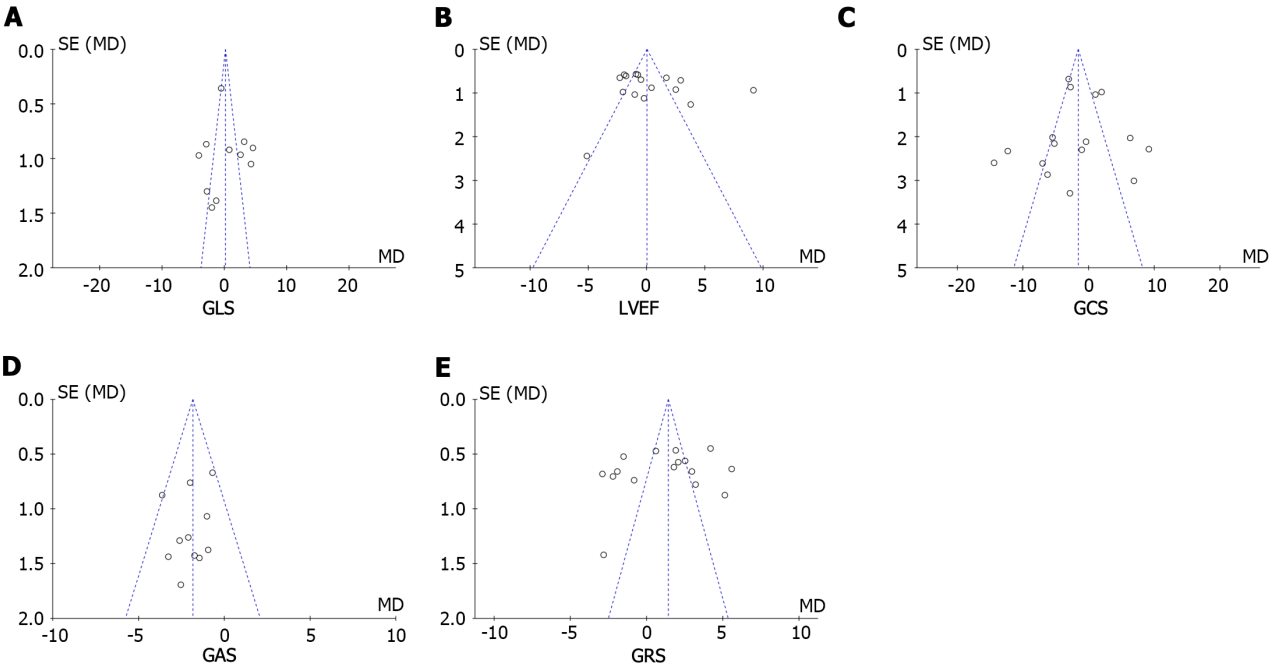

Figure 7 Risk of bias assessment for global longitudinal strain, left ventricular ejection fraction, global circumferential strain, global area strain, and global radial strain. A: Global longitudinal strain; B: Left ventricular ejection fraction; C: Global circumferential strain; D: Global area strain; E: Global radial strain. GLS: Global longitudinal strain; LEVF: Left ventricular ejection fraction; GCS: Global area strain; GAS: Global area strain; GRS: Global radial strain.

Conventional echocardiography is the most frequently used imaging approach for measuring and evaluating left ventricular function[16]. Evaluation of left ventricular function according to the LVEF has been widely used in the clinic [17]. The use of strain imaging of the myocardium opens a new window for the study of myocardial mechanics. Strain measurement according to the myocardial mechanics of the left ventricle can quickly and effectively evaluate changes in myocardial contractility[18]. Speckle tracking imaging (STI) tracks myocardial tissue signals frame by frame through the principle of "block matching", without significant displacement between adjacent frames, and can evaluate myocardial motion and quantify changes in cardiac function without angle dependence[19]. With the emergence of 2D-STI, the abovementioned left ventricular myocardial strain parameters can be measured quantitatively[20]. 3D-STI combines real-time three-dimensional echocardiography and STI and can track myocardial tissue signals in three-dimensional space without the limitation of planes, which compensates for the deficiency of 2D-STI[21]. This is the first meta-analysis evaluating the clinical utility of the 3D-STI for assessing LVMCF in patients with DM.

The consensus is that the LVEF is the stroke volume after the standardized change in left ventricular volume. It is a frequently used parameter for the clinical assessment of LVSF. At present, magnetic resonance imaging is considered the gold standard for detecting LVSF[22]. The findings of this meta-analysis implied that there were anomalous alterations in left ventricular myocardial mechanics in DM patients without a significant decrease in LVEF. In addition, 3D-STI can yield parameters such as the GRS, GLS, GAS, GCS, and 3D-strain. Saeedi *et al*[23] reported that GLS, GRS, GCS and GAS

were decreased in DM compared with those in the control group. Baber *et al*[24] reported that there was no marked difference in GGS or GRS between DMs and controls, but the GLS and GAS were considerably lower than those in controls.

The consequences of our meta-analysis indicated that the GLS, GRS, GCS, and GAS in DMs were decreased compared with those in controls, suggesting that the LVSF in DMs was impaired compared with that in controls. The decreases in GLS and GAS were more obvious, possibly because the left ventricular wall is composed of three layers of myocardial fibers. The subepicardial myocardial fibers are arranged counterclockwise oblique in the direction of the left ventricular longitudinal axis, approximately circular in the middle layer of the ventricular wall, and clockwise in the longitudinal axis to the innermost layer, namely, the subendocardial layer. The diversity of myocardial fiber arrangement determines the complexity of left ventricular three-dimensional motion[25]. GLS represents the ability of the heart to move in the long axis direction and is caused by the contraction of longitudinal muscle fibers under the endocardium. These muscle fibers have the characteristics of strong contractility and high demand for oxygen, which may be the reason why longitudinal strain is more sensitive than that in other directions of the left ventricle during mild hypoperfusion of the subendocardial myocardium in the early stage of diabetes[26]. The GRS and GCS are mainly affected by the contractility of annular fibers in the middle layer of the myocardium. The GAS reflects the rate of change in the endocardial area from the initial area to the area after deformation; this metric is a strain index integrated with longitudinal and circumferential strain, is inversely proportional to the radial strain, and can be regarded as the composite of GLS and GRS[27]. As reported previously, the GAS has the best correlation with LVSF[28]. Wang *et al*[29] suggested that the GAS could offer a more precise basis for quantifying global and local myocardial function with good reproducibility. Chen *et al*[30] reported that there was a negative correlation between GAS and GLS and between GAS and hemoglobin A1c, among which GAS had the strongest correlation. Additionally, another study confirmed that the GAS can provide a more accurate basis and good reproducibility for quantifying global and local myocardial function[31].

The present meta-analysis has several limitations. Firstly, the studies included in this meta-analysis included diseases such as hypertension or obesity in DM patients and good or poor control of blood glucose levels in DM patients. Therefore, there might be selection bias. Second, only 8 articles were involved in this study, and the sample size was rather small, so the results might be affected. However, we strictly established the selection criteria for the articles and strictly evaluated the quality of the studies to improve the overall quality of the meta-analysis and the credibility of the results.

CONCLUSION

In conclusion, the 3D-STI might be used to precisely calculate early LVSF in patients with DM. The measurement of left ventricular strain in DM patients through 3D-STI could estimate the LVSF damage in patients with early diabetes.

ARTICLE HIGHLIGHTS

Research background

Diabetic cardiomyopathy is a chronic complication, which is a critical reason of poor prognosis and even death in patients with diabetes mellitus (DM). Additionally, numerous reports have implied that DM could raise the occurrence of heart disorder, hypertension, and other illnesses, and could worsen coronary artery illness. Therefore, early detection of left ventricular systolic function (LVSF) damage in DM, necessary treatment is especially essential. Three-dimensional speckle tracking technique (3D-STI) is of beneficial worth in the assessment of primary or secondary LVSF. However, the 3D-STI evaluating capability of left ventricular myocardial contractile function (LVMCF) in DM remains uncertain and further reports are needed.

Research motivation

To explore the application value of 3D-STI in assessing LVMCF in DM by meta-analysis.

Research objectives

To investigate the assessment of 3D-STI in estimating early left ventricular systolic dysfunction in DM by meta-analysis. 3D-STI provides a feasible and accurate new technique for clinical measurement of LVSF in left ventricular caused by DM, which might play an important role in the evaluation of cardiac function injury.

Research methods

We carried out a meta-analysis to evaluate myocardial function in patients with DM compared with controls according to myocardial strain attained by 3D-STI. We searched he PubMed, Embase, Scopus databases, and the Cochrane library from the initial accessible time to 29 April 2023. PRISMA guidelines were used. Data for meta-analysis were pooled using a random-effects model. We extracted data and used the Cochrane "Risk of bias" tool to assess methodological quality. Effect was presented as mean difference with 95% confidence interval using RevMan 5.3. The current study is the first meta-analysis to report that 3D-STI could precisely assess early left ventricular systolic dysfunction in DM.

Research results

The findings of this meta-analysis implied that there existed anomalous alterations in left ventricular myocardial mechanics in DM without a significant decrease in LVEF. In addition, 3D-STI could obtain parameters such as GRS, GLS, GAS, GCS, 3D-Strain and so on. Among them, the decrease of GLS and GAS was more obvious, which may be since the left ventricular wall is composed of three layers of myocardial fibers: The subepicardial myocardial fibers are arranged counterclockwise oblique in the direction of the left ventricular longitudinal axis, approximately circular in the middle layer of the ventricular wall, and clockwise in the longitudinal axis to the innermost layer, namely the subendocardial layer.

Research conclusions

Our data provided the first evidence for the essential role of 3D-STI in assessing the early left ventricle systolic dysfunction in DM precisely. The assessment of left ventricular strain in DM patients through 3D-STI might estimate the damage of LVSF in DM in the early stage.

Research perspectives

We believe that with the continuous improvement of computer and three-dimensional ultrasound technology, the shortcomings will be overcome, and 3D-STI is expected to become the gold standard for clinical non-invasive determination of LVSF.

FOOTNOTES

Author contributions: Li Z and Qian Y were responsible for the study concept and designed the systematic review protocol; Qian Y and Huang Y performed the study selection and data extraction; Li Z and Fan CY performed the statistical analyses; Qian Y and Huang Y prepared the outlines and wrote the manuscript; and all the authors have contributed to the completion of this paper.

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LETTER TO THE EDITOR

Metabolic syndrome's new therapy: Supplement the gut microbiome

Yong-Wei Xu, Jun Tian, Yan Song, Ba-Cui Zhang, Jing Wang

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Abstract

This letter to the editor discusses the publication on gut microbiome supplementation as therapy for metabolic syndrome. Gut microbiome dysbiosis disrupts intestinal bacterial homeostasis and is related to chronic inflammation, insulin resistance, cardiovascular diseases, type 2 diabetes mellitus, and obesity. Previous research has found that increasing the abundance of beneficial microbiota in the gut modulates metabolic syndrome by reducing chronic inflammation and insulin resistance. Prebiotics, probiotics, synbiotics, and postbiotics are often used as supplements to increase the number of beneficial microbes and thus the production of short-chain fatty acids, which have positive effects on the gut microbiome and metabolic syndrome. In this review article, the author summarizes the available supplements to increase the abundance of beneficial gut microbiota and reduce the abundance of harmful microbiota in patients with metabolic disorders. Our group is also researching the role of the gut microbiota in chronic liver disease. This article will be of great help to our research. At the end of the letter, the mechanism of the gut microbiota in chronic liver disease is discussed.

Key Words: Gut microbiome; Metabolic syndrome; Diabetes mellitus; Short-chain fatty acids; Chronic liver disease

Core Tip: I am writing to express my appreciation for the enlightening study titled "Gut microbiome supplementation as therapy for metabolic syndrome" recently published in your esteemed journal. The findings presented in this research shed light on the pivotal role of the gut microbiome in modulating metabolic syndrome, emphasizing the potential therapeutic impact of supplementation. The paper delves into the diverse array of supplements, including probiotics, synbiotics and postbiotics, and their distinct effects on the gut microbiome and its association with metabolic syndrome. I believe that this study will further investigations, inspire clinical trials, and foster the development of targeted interventions to enhance metabolic health through the modulation of the gut microbiome. Our research group is also researching the role of the gut microbiota in chronic liver disease. This article will be of great help to our research.

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TO THE EDITOR

Dear Editor, we read with great interest the recently published review paper by Mc Anto Antony, entitled "Gut microbiome supplementation as therapy for metabolic syndrome", in the *World Journal of Diabetes*[1]. The gut microbiota may have a significant role in controlling the host's health. Even though gut microbes have been studied for several decades, the investigation into the purposes of those microorganisms has attracted significant interest outside of the realm of traditional diseases associated with infection. This systematic review of recent evidence from some mice and human model experiments concluded that microbiome supplementation improves people's gut microbiomes that suffer from metabolic syndrome. They produce beneficial metabolites, such as short-chain fatty acids (SCFAs), including butyrate, acetate, and propionate which can improve insulin sensitivity and the patient's body weight[2]. Acetate, butyrate, and propionate are formed because of the intestinal microflora's fermentative action on fibre from the diet. The microbiota in the gut transforms the primary bile acids into bile acids, which may further increase the synthesis of cyclic adenosine monophosphate and enhance insulin sensitivity[3].

We agree with that opinion in this review. Previous studies similarly reported that microbiome supplementation increases the gut microbes' abundance of benefits and reduces the prevalence of dangerous bacteria in people with metabolic syndrome and diabetes. Endotoxemia and chronic inflammation can result from type 2 diabetes mellitus weakening the barrier between the intestines and allowing gram-negative microbes to get into the circulatory system[4]. Gut microbiome supplements, for example, probiotics, synbiotics, or postbiotics, affect the gut microbiome and metabolic syndrome by increasing the abundance of beneficial microbiota; therefore, these supplements could reduce insulin resistance and chronic inflammation to modify the metabolic syndrome[3].

Prebiotics provide a multitude of health effects, one of which is immunological regulation *via* the creation of more immune globulin and interleukins (IL) and a decrease in inflammatory IL[5]. Additionally, the SCFAs may lower gut pH, which will stop harmful bacteria from growing. These elements can enhance insulin sensitivity, enhance the condition of the gut, as well as decrease the generation of cytokines that are associated with inflammation[6]. Among probiotics, the two most associated bacteria are Lactobacillus and Bifidobacterium. It has been discovered that probiotics affect the expression of genes and proteins linked to inflammation[7]. Among the beneficial bacteria crucial in preserving this barrier in the intestine are *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*. *Facecalibacterium prausnitzii* is linked to decreased inflammation and creates SCFA butyrate. Bifidobacteria enhance insulin sensitivity, enhance gut health, and reduce the generation of cytokines associated with inflammation[8]. From previous research, we found that probiotics, synbiotics, or postbiotics are three substances that can lead to the production of SCFAs, in the gastrointestinal tract improve health and promote intestinal barrier integrity.

Three SCFAs possess an impact on energy homeostasis and metabolic processes, and they may positively influence the functioning of muscle in the skeleton, the tissue of the liver, and fatty tissue. These SCFAs not only are of importance in gut health and as signaling molecules but also might directly affect metabolism[9]. SCFAs in the gut, maybe they can improve the health of the gastrointestinal tract, and then they also can protect against inflammation and most importantly promote intestinal barrier integrity.

SCFAs are produced in the distal intestine during the fermentation of indigestible meals. In the caecum, ileum, and colon, the ratio of acetate to propionate to butyrate is around 3:1:1[10]. Since the liver and colon are normally where butyrate and propionate are processed, they primarily impact the functioning of the local gastrointestinal tract and liver [11]. such as GPR41 and GPR43 were G-protein-coupled receptors, in the distal gut are bound by SCFAs, influencing satiety and glucose homeostasis, and producing the gut hormones peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) [12]. High concentrations of acetate and low levels of propionate and butyrate enter the bloodstream and can have an immediate impact on the function and metabolism of substrates in the muscles, liver, and peripheral fat tissue. Acetate and butyrate might influence skeletal muscle glucose metabolism in an adenosine monophosphate-activated protein kinase (AMPK)-dependent manner, this could promote the uptake of glucose (via the glucose transporter type 4; GLUT4) and the subsequent retention of glycogen (perhaps through a process that involves GPR41/GPR43)[13]. Through elevated systemic levels of gut-derived PYY and GLP-1, SCFAs may also indirectly influence muscle insulin susceptibility and the

breakdown of glucose. This could impact the muscle's response to insulin and absorption of glucose, improving tissue insulin tolerance and glucose management[14].

Some proinflammatory cytokines such as IL-6, IL-1β, IL-8, and tumour necrosis factor-α (TNF-α) may be affected by SCFAs in epithelial cells, and they are also promoted by enhancing nuclear factor kappa-B activation in Toll-like receptors ligand responses[15]. SCFAs can also inhibit the production of proinflammatory cytokines, such as TNF-α, in neutrophils [16]. SCFAs regulate dendritic cell activities, which in turn control the immunological response by influencing T-cell interaction as well as cytokine secretion.

Butyrate and propionate inhibit the activation of bone marrow-derived stem cells by suppressing the LPS-induced expression of the costimulatory molecule CD40 and the secretion of IL-6 and IL-12p40[16]. They can inhibit proinflammatory mediators, including nitric oxide (NO), IL-6, and IL-12, but they do not affect the production of TNF-α or monocyte chemotactic protein 1. Growing evidence points to SCFAs' regulatory role in both adaptive and innate immune cells, among other immune system cell types.

The potential role of increasing SCFAs as a metabolic tool to prevent insulin resistance and associated cardiometabolic risk factors is increasing. It is still unknown how these findings may affect human metabolism and clinical relevance because most of the data come from *in vitro* and animal research. This may be important for future research. Ingesting complex carbs stimulates the microbiota to produce SCFAs, a helpful strategy to stop glucose metabolism and potentially delay the onset of insulin resistance. Long-term well-controlled human intervention studies are needed to clarify the role of SCFAs in the control of body weight and insulin sensitivity and to define the mechanism of action of SCFAs in organisms. Supplementing with gut microbiota can help treat the metabolic syndrome.

When probiotics are supplemented, side effects may be caused. Probiotics are not released in the stomach but play a role in the intestine. When excessive probiotics are supplemented, it will cause intestinal dysfunction. In addition, in some patients with severely damaged intestinal barrier, bacteria easily break through the intestinal barrier into the blood, causing infection. However, randomised, large-scale, high-quality research and clinical trials should be developed to evaluate the use of SCFAs to alter the gut microbiome and affect different metabolic illnesses. The side effects of bacteria also need to be considered, which will be another focus of research. Moreover, additional beneficial strains with effects on the gut microbiome need to be identified for the treatment of other diseases.

According to this review, gut-healthy bacteria enhance insulin sensitivity and reduce the release of cytokines associated with inflammation. Therefore, in our research centre, we consider that SCFAs may affect the composition of the gut microbiome and its functions can affect chronic liver disease most likely by regulating innate and adaptive cells; however, further investigation is needed.

FOOTNOTES

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